Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 57 DECEMBER, 1944 No. 3

SECTION MEETINGS

IOWA
State University of Iowa November 28, 1944

14790

Inflammation. VII. Selective Inhibitory Action of Tuberculo-Carbohydrate and Phosphatide on Cellular Cathepsins from Tuberculous Tissues.

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In an enzymologic investigation designed to throw light on the mechanism of the specific cytotoxic effect of tuberculin *in vitro*, we observed that the purified protein derivative of tuberculin (PPD) inhibits to an equal degree the proteinases (Cathepsin II) from organs of normal animals and those infected with either virulent or nonvirulent tubercle bacilli.[1] Tuberculin (OT), on the other hand, had but slight effect, in tissue culture, on cells from normal animals, but severely injured cells derived from animals infected with either strain of tubercle bacilli.[2] These experiments therefore did not throw any new light upon the mechanism of the tuberculin reaction. In the present communication we report upon experiments with carbohydrate and phosphatide fractions of the tubercle bacillus, *M. tuberculosis*, H-37, in which the results were positive and significant.

Experimental. The cathepsin solutions were prepared as previously described[3] by the methods of Bergmann and Fruton[4] from livers of normal rabbits (Series A), those infected with a strain (R_1) of tubercle bacilli of low virulence (Series B), those infected with a virulent, Ravenel culture (Series C), and those from animals first injected with an R_1 culture and later reinfected with the Ravenel strain (Series D). For purposes of comparison, normal beef spleen cathepsin and papain, purified by the method of Greenberg,[5] were also tested.

The enzyme preparations were dialyzed for 24 hours at 7°C against 1% NaCl and used immediately, or, in some cases, were preserved in a frozen state at −70° for several days. These dialyzed extracts show increased NPN and lower protein nitrogen, but their hydrolytic activity (unit of enzyme per mg of protein N) remains constant up to about one

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1 Weiss, C., and Halliday, N., *Arch. Path.*, 1944, **37**, 272.

2 Moen, J. K., and Swift, H. F., *J. Exp. Med.*, 1936, **64**, 339.

3 Weiss, C., and Halliday, N., *J. Immunol.*, 1944, **49**, 251.

4 Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, 1939, **130**, 19.

5 Winnick, T., Davis, A. R., and Greenberg, D. M., *J. Gen. Physiol.*, 1939, **23**, 301.

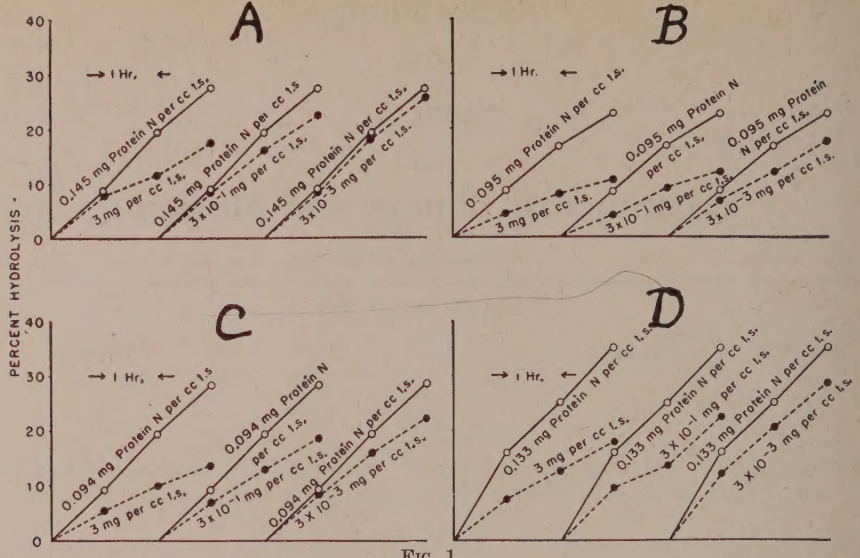

FIG. 1.

Hydrolysis of benzoyl-*l* arginineamide (BAA) in the presence and absence of tuberculo-phosphatide by cathepsin II prepared from livers of normal and tuberculous rabbits. (Series A, B, C, and D are described in the text under "Experimental.")

week. All hydrolyses were carried out simultaneously in the presence and absence of the phosphatide or carbohydrate, as previously described.[1] The substrate employed was benzoyl-*l*-arginineamide (BAA); the enzymes were activated by cysteine and buffered with 0.05 M citrate, pH 4.9. The enzyme concentrations were selected to give about 20 to 30% substrate hydrolysis in a 3-hour period, using the Grassmann and Heyde titration method,[6] where an increase of 1 cc of 0.01 M alcoholic KOH represents 100% splitting of a peptide bond.

The tuberculo-carbohydrate ("B.M. Supt. II") was prepared by Dr. Sidney Raffel of Stanford University[7] according to the method of Heidelberger and Menzel.[8] The tubercle bacilli were grown on Long's synthetic medium, the defatted organisms pooled, and the carbohydrate extracted with acidified water. This polysaccharide precipitated with homologous immune horse serum in a dilution of one to one million.

The tuberculo-phosphatide (H-37; A-3) was obtained from Dr. R. J. Anderson of Yale University. The concentrations which could be employed were governed by its low solubility in water. The levels selected for this substance as well as for the carbohydrate were approximately: 3, 3×10^{-1}, and 3×10^{-3} mg per cc of test solution. The final mixtures of phosphatide with the two higher levels were opaque, but since the material was readily soluble in alcohol, it was possible to obtain good titration end-points.

In general, phospholipids are relatively unstable. Losses of 20 to 25% were observed by Halliday[9] and Boyd[10] when alcohol-ether extracts of tissues were stored up to 3 months at room temperature even in the dark. It was thought that aqueous suspensions could be preserved in the ice-box up to 2 to 3 weeks, but it was found that after only 1 week there was loss of inhibitory power. In subsequent experiments, therefore, solutions were freshly prepared for each experiment or used after only 24 hours of storage.

Three types of experiments were performed with phosphatide: (1) the substrate, cysteine-

[6] Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, 1929, **183**, 32.

[7] Raffel, S., *Stanford Med. Bull.*, 1943, **1**, 209.

[8] Heidelberger, M., and Menzel, A. E. O., *J. Biol. Chem.*, 1937, **118**, 79.

[9] Halliday, N., *J. Nutr.*, 1938, **16**, 285.

[10] Boyd, E. M., *J. Biol. Chem.*, 1937, **121**, 485.

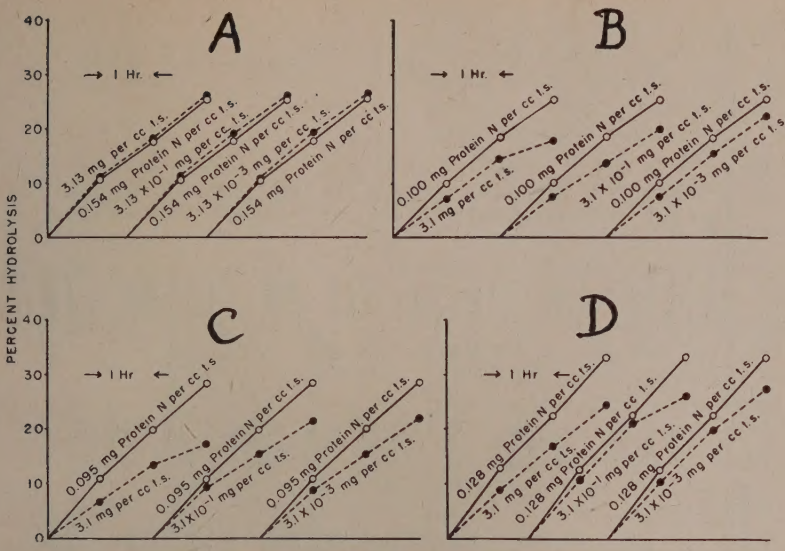

FIG. 2.

Hydrolysis of benzoyl-*l*-arginineamide (BAA) in the presence and absence of tuberculo-carbohydrate by cathepsin II prepared from livers of normal and tuberculous rabbits. (Series A, B, C, and D are described in the text under "Experimental.")

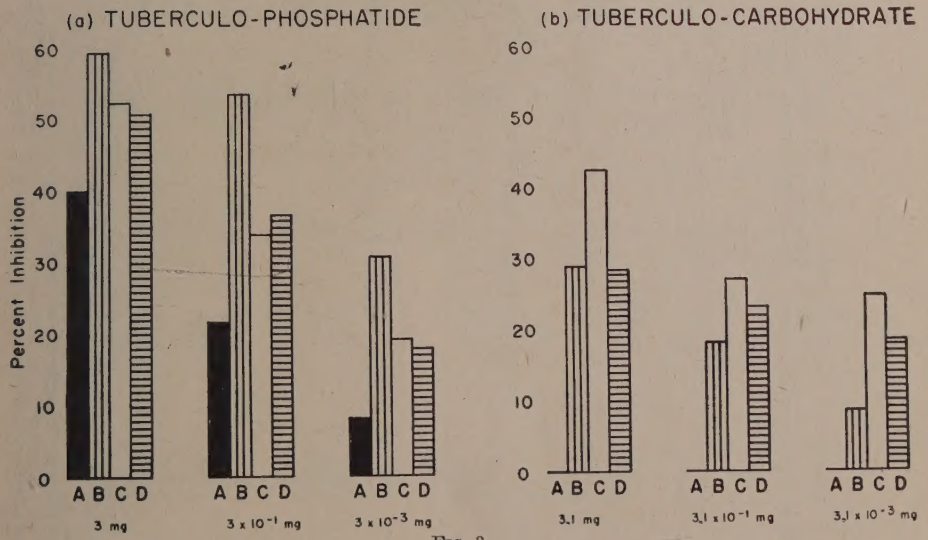

FIG. 3.

Percentages of inhibition of hydrolysis of BAA by (a) tuberculo-phosphatide and (b) tuberculo-carbohydrate. Results are based upon a three-hour interval. Concentrations of inhibitor per cc of test solution are shown under the columns.

buffer solution, and phosphatide were mixed and warmed to 40° just before addition of enzyme and beginning of the 3-hour hydrolysis period; (2) the bath temperature was lowered to 30° and hydrolyses continued for 24 hours, with titrations at zero, 5 and 24 hours; and

TABLE I.

Effect of Tuberculo-phosphatide on the Hydrolysis of Benzoyl-*l*-arginineamide (BAA) by Cathepsin II Prepared from Livers of Normal Rabbits and Those Infected with Virulent and Non-Virulent Tubercle Bacilli.

Conc. of phosphatide mg per cc test sol.	Conc. of enzyme	K × 10⁴		C × 10³		Unit		Unit per mg		% decrease
		Without phospha-tide	With phospha-tide	Without phospha-tide	With phospha-tide	Without phospha-tide	With phospha-tide	Without phospha-tide	With phospha-tide	
				Series A. Normal Rabbits.						
0.0	0.291	15.6		5.35		0.374		2.67		
3.0	0.145	7.76	4.64	5.34	3.19	0.375	0.627	2.67	1.60	40.1
3 × 10⁻¹	0.145		6.09		4.19		0.478		2.09	21.7
3 × 10⁻³	0.145		7.13		4.91		0.408		2.45	8.24
				Avg: 5.35		Avg: 0.375		Avg: 2.67		
				Series B. Rabbits Infected with a Non-Virulent (R_1) Strain of Tubercle Bacilli.						
0.0	0.189	14.4		7.59		0.263		3.80		
3.0	0.095	6.21	2.71	6.56	2.86	0.305	0.700	3.28	1.43	59.6
3 × 10⁻¹	0.095		3.11		3.29		0.609		1.64	53.7
3 × 10⁻³	0.095		4.64		4.90		0.408		2.45	30.8
				Avg: 7.08		Avg: 0.284		Avg: 3.54		
				Series C. Rabbits Infected with a Virulent Ravenel Strain of Bovine Tubercle Bacilli.						
0.0	0.188	13.7		7.31		0.274		3.66		
3.0	0.094	8.06	3.56	8.58	3.79	0.233	0.528	4.29	1.89	52.5
3 × 10⁻¹	0.094		4.93		5.25		0.381		2.63	33.9
3 × 10⁻³	0.094		6.05		6.45		0.310		3.22	19.1
				Avg: 7.95		Avg: 0.254		Avg: 3.98		
				Series D. Rabbits Immunized with an R_1 Strain and Reinfected with a Ravenel Strain.						
0.0	0.265	18.0		6.80		0.294		3.40		
3.0	0.133	10.5	4.79	7.90	3.61	0.253	0.553	3.95	1.81	50.8
3 × 10⁻¹	0.133		6.18		4.67		0.429		2.33	36.7
3 × 10⁻³	0.133		8.03		6.06		0.330		3.03	17.7
				Avg: 7.35		Avg: 0.274		Avg: 3.68		

TABLE II.

Effect of Tuberculo-phosphatide on the Hydrolysis of Benzoyl-*l*-argininеamide (BAA) by Purified Papain and by Cathepsin II Prepared from Normal Beef Spleen.

Conc. of phosphatide mg per cc test sol.	Conc. of enzyme	K × 10⁴		C × 10³		Unit		Unit per mg		% decrease
		Without phosphatide	With phosphatide	Without phosphatide	With phosphatide	Without phosphatide	With phosphatide	Without phosphatide	With phosphatide	
				Purified Papain.						
0.0	0.0155	22.5		14.6		0.0137		72.8		
3.0	0.0077	11.6	12.4	15.0	16.0	0.0133	0.0125	75.0	80.0	0.0
3 × 10⁻³	0.0077		11.5		14.9		0.0134		74.5	0.0
				Avg: 14.8		Avg: 0.0135		Avg: 73.9		
				Beef Spleen Cathepsin.						
0.0	0.136	13.8		10.2		0.197		5.09		
3.0	0.068	7.01	3.87	10.3	5.69	0.194	0.351	5.16	2.85	44.4
3 × 10⁻¹	0.068		5.78		8.51		0.235		4.25	17.2
3 × 10⁻³	0.068		7.46		11.0		0.182		5.49	0.0
				Avg: 10.3		Avg: 0.196		Avg: 5.13		

(3) the cysteine-buffer solution and phosphatide were warmed to 40°, the enzyme then added, and the material held at this temperature for 2 hours before the substrate was added and the titrations begun. The hydrolysis periods were 3 hours. The latter was the only type of experiment employed with the carbohydrate.

In the first method there was little or no effect of phosphatide on the enzyme. If digestion was allowed to proceed at 30° for 24 hours, the rate of enzyme activity was decreased, but only the highest level of phosphatide exerted any inhibitory action. The third method proved of greatest value. Graded differences were observed with graded levels of phosphatide. The results with the carbohydrate were not so closely correlated with the concentration of carbohydrate, but significant inhibition was obtained with enzymes prepared from tuberculous organs, whereas there was no effect whatever on those from normal liver or from beef spleen.

The method of calculating activity constants and units of enzyme was that of Bergmann, Fruton, and coworkers.[11] In the experiments without inhibitor, first-order kinetics were found. In the presence of higher levels of phosphatide or carbohydrate, however, the K values were not constant at the various time intervals.

Results. Results are shown in Figs. 1 to 3 and Tables I to IV. For the sake of comparison with data without inhibitor, the various constants for the time interval of zero to 180 minutes were employed. It is possible that a two-phase system is present. The inhibition is not due to the presence of end-products of the hydrolysis since a pure substrate was employed and the phosphatide yields the following substances[12] after saponification with dilute alcoholic KOH: alcoholic soap solution of the fatty acids, a small amount of free glycerol and phosphoric acid, and an alcohol-insoluble residue consisting of a mixture containing a complex organic phosphoric acid and a neutral carbohydrate (man-

[11] Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, 1941, **138**, 231.

[12] Anderson, R. J., and Roberts, E. G., *J. Am. Chem. Soc.*, 1930, **52**, 5023.

TABLE III.

Effect of Tuberculo-carbohydrate on the Hydrolysis of Benzoyl-*l*-arginineamide (BAA) by Cathepsin II Prepared from Livers of Normal Rabbits and Those Infected with Virulent and Non-Virulent Tubercle Bacilli.

Conc. of carbohydrate mg per cc test sol.	Conc. of enzyme	K × 10⁴		C × 10³		Unit		Unit per mg		% decrease
		Without carbohydrate	With carbohydrate	Without carbohydrate	With carbohydrate	Without carbohydrate	With carbohydrate	Without carbohydrate	With carbohydrate	
				Series A. Normal Rabbits.						
0.0	0.308	14.4		4.68		0.427		2.34		
3.1	0.154	7.04	7.23	4.57	4.70	0.438	0.426	2.29	2.35	0.0
3.1 × 10⁻¹	0.154		7.20		4.68		0.428		2.34	0.0
3.1 × 10⁻³	0.154		7.27		4.72		0.424		2.36	0.0
				Avg: 4.63		Avg: 0.433		Avg: 2.32		
				Series B. Rabbits Infected with a Non-Virulent (R_1) Strain of Tubercle Bacilli.						
0.0	0.200	12.2		6.10		0.328		3.05		
3.1	0.100	7.01	4.67	7.00	4.67	0.286	0.429	3.50	2.33	29.0
3.1 × 10⁻¹	0.100		5.36		5.35		0.374		2.68	18.3
3.1 × 10⁻³	0.100		6.00		5.99		0.334		3.00	8.5
				Avg: 6.55		Avg: 0.307		Avg: 3.28		
				Series C. Rabbits Infected with a Virulent Ravenel Strain of Bovine Tubercle Bacilli.						
0.0	0.190	15.6		8.21		0.244		4.11		
3.1	0.095	8.13	4.58	8.54	4.82	0.234	0.415	4.27	2.41	42.5
3.1 × 10⁻¹	0.095		5.81		6.10		0.328		3.05	27.2
3.1 × 10⁻³	0.095		6.00		6.30		0.318		3.15	24.8
				Avg: 8.38		Avg: 0.239		Avg: 4.19		
				Series D. Rabbits Immunized with an R_1 Strain and Reinfected with a Ravenel Strain.						
0.0	0.256	18.5		7.21		0.277		3.61		
3.1	0.128	9.73	6.78	7.60	5.30	0.263	0.377	3.80	2.65	28.6
3.1 × 10⁻¹	0.128		7.30		5.70		0.351		2.85	23.2
3.1 × 10⁻³	0.128		7.73		6.04		0.331		3.02	18.6
				Avg: 7.41		Avg: 0.270		Avg: 3.71		

TABLE IV.

Effect of Tuberculo-carbohydrate on the Hydrolysis of Benzoyl-*l*-argininеamide (BAA) by Cathepsin II Prepared from Normal Beef Spleen.

Conc. of carbohydrate mg per cc test sol.	Conc. of enzyme	K × 10⁴		C × 10³		Unit		Unit per mg		% decrease
		Without carbohydrate	With carbohydrate	Without carbohydrate	With carbohydrate	Without carbohydrate	With carbohydrate	Without carbohydrate	With carbohydrate	
0.0	0.142	13.2		9.26		0.216		4.63		
3.1	0.071	6.43	6.62	9.04	9.30	0.221	0.215	4.52	4.65	0.0
3.1 × 10⁻¹	0.071		6.59		9.26		0.216		4.63	0.0
3.1 × 10⁻³	0.071		6.84		9.61		0.208		4.81	0.0
				Avg: 9.15		Avg: 0.219		Avg: 4.58		

ninositose). According to Heidelberger and Menzel[8] the carbohydrate is obtained as a complex mixture of polysaccharides made up of *d*-arabinose and *d*-mannose.

It is evident that tuberculo-phosphatide definitely exerts a much greater inhibitory action on cathepsin derived from tuberculous tissues of rabbits than on similar enzyme solutions prepared from livers of normal animals. Whether or not there is any significance to the observation that the greatest inhibition was observed with cathepsin prepared from the livers of animals infected with the non-virulent R_1 strain remains to be determined.

The results with tuberculo-carbohydrate are even more striking: there was no inhibition whatever exerted upon normal liver cathepsin and very strong inhibition upon the proteinases prepared from tuberculous tissues. The significance of the apparent maximal inhibitory values in the case of tissues derived from animals infected for the first time with virulent, Ravenel tubercle bacilli, will be investigated.

Discussion. Several investigators, including Moen,[2] have come to the conclusion that the specific toxic action of tuberculin upon hypersensitive tissues is probably not the result of an antigen-antibody reaction. The discovery, in the present communication, of a selective inhibitory action of tuberculo-carbohydrate and tuberculo-phosphatide upon endocellular enzymes (Cathepsin II) derived from tissues of animals infected with tubercle bacilli throws new light upon this mechanism and suggests an enzymological explanation, as follows: Since cathepsin is the enzyme which is concerned with the processes of cellular growth and repair, inhibition thereof leads to injury of the cells. Tuberculin is more toxic for tuberculous than for normal cells in tissue culture because two of its important constituents (phosphatide and carbohydrate) exert a selective inhibitory action upon the proteinases of the former.

Our observations may also throw light on the mechanism of caseation and softening. As pointed out by Jobling and Petersen,[13] "Caseation in tuberculosis is a form of coagu-

[13] Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, **19**, 383.

lation necrosis in which the dead tissues rarely undergo autolysis, except as a result of secondary infection." It is quite likely, therefore, that inhibition of autolysis is accomplished by the carbohydrate and phosphatide fractions of the tubercle bacilli.

It is also possible that the development of an increased susceptibility to the inhibitory action of the products of the tubercle bacillus which comes about as a result of infection is a defense mechanism since, as shown by Lurie,[14] tubercle bacilli usually die in caseous but grow in the softened areas. Furthermore, the former may undergo calcification and healing, whereas in the latter, the bacilli grow and from there disseminate to other sites.

Conclusions. The carbohydrate and phosphatide fractions of the tubercle bacillus (*M. tuberculosis*, H-37) exert a selective inhibitory action upon the endocellular enzyme, Cathepsin II, of tuberculous tissue. This phenomenon may help to explain the cytotoxic action of tuberculin *in vitro* and the failure of autolysis of caseous, tuberculous tissue.

[14] Lurie, M. B., *J. Exp. Med.*, 1933, **57**, 181.

14791 P

The Endotoxin of the Cholera Vibrio: Isolation and Properties*

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Though it is generally agreed that the cholera vibrio contains a potent endotoxin, the nature of the toxin is by no means clear. The isolation of a toxic protein has been reported by some workers such as Galeotti,[1] Sanarelli,[2] and Hahn and Hirsch.[3] More recently the trichloracetic acid extraction method of Boivin[4] has been applied to the cholera vibrio by Checcacci,[5] Raynal, Lieou, and Feissole,[6] Damboviceanu and Barber[7] and Gallut,[8] all of whom have reported successful extraction of a toxic fraction. It has been assumed by these workers that the active material is a polysaccharide-lipid complex, though in no instance is this conclusion supported by unequivocal evidence.

In the course of a study of active immunity to infection with *Vibrio choleræ* we have had occasion to isolate the toxic principle from both Inaba and Ogawa types and partially purify it. Toxic solutions of the vibrio cell substance have been prepared in the following ways: (a) The cells are readily disintegrated in 4-5 hours by high speed grinding with sand, and the cellular debris is spun off, leaving a toxic opalescent supernate. (b) The cells may be dissolved in 6 M urea to give a similar toxic solution. (c) The cells may be digested with pepsin 3-5 days without inactivation of the toxicity, and the insoluble material removed by centrifugation to leave a toxic supernate. (d) The intact cells may be extracted in the cold with M/2 trichloracetic acid, all the toxicity going into solution. (e) Lyophilized cells may be extracted with methyl alcohol, ethyl alcohol, chloroform, or ethyl ether in a Soxhlet apparatus to give toxic

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

[1] Galeotti, G., *Centralbl. f. Bakt., I Abt. Orig.*, 1912, **67**, 225.

[2] Sanarelli, G., *Ann. Inst. Pasteur*, 1920, **34**, 370.

[3] Hahn, M., and Hirsch, J., *Centralbl. f. Bakt., I Abt. Orig.*, 1927, **104**, 211; *Klin. Woch.*, 1927, **6**, 312; *Z. f. Hyg. u. Infektionskr.*, 1929, **110**, 355.

[4] *Cf.* Boivin, A., and Mesrobeanu, L., *Rev. Immunol.*, 1935, **1**, 553.

[5] Checcacci, L., *Boll. Instituto Sieroterap. Milanese*, 1939, **18**, 391.

[6] Raynal, J., Lieou, Y. C., and Feissole, L., *Rev. Immunol.*, 1939, **5**, 317; *ibid.*, 1940, **6**, 132.

[7] Damboviceanu, A., and Barber, G., *C. R. Soc. Biol.*, 1940, **133**, 501.

[8] Gallut, J., *Ann. Inst. Pasteur*, 1943, **69**, 123.

extracts. Attempts to extract the toxicity from the intact cells with glycols have not been successful.

Of these the first method used was that of trichloracetic acid extraction. When the neutralized extract was dialyzed through cellophane to remove salt it was found that the activity appeared in the dialysate. The dialysis was rapid for toxicity was no longer detectable after 3-4 hours when carried out against running water. The toxicity may also be separated from the ground cells by dialysis though the process is slow, requiring 10 days in the refrigerator with daily changes of water. These observations are consistent with those of others, most recently Basu, Chaudhury, and Basu[9] and Banerjee,[10] who have reported that the toxicity is dialyzable.

The addition of 3-5 volumes of ethyl alcohol to the trichloracetic acid extract resulted in the appearance of a flocculent precipitate, which was shown to be polysaccharide, while the toxicity remained in solution. If polysaccharide is precipitated from concentrated extracts, the precipitate may contain some toxicity as an impurity but is readily purified.

When the filtrate from alcoholic precipitation was concentrated by evaporation under lamp and fan, a yellow oil separated out which contained most of the toxicity and had a mouse MLD of 0.1 cc. It appeared to be a mixture of alcohol and lipids. Similar material has been described by Raynal, Lieou, and Feissole.[6] Although studied somewhat further, preparations of this type did not appear to be promising because of the difficulty of separating the toxicity from trichloracetate.

The solubility of the activity in alcohol led to attempts to extract it directly from the vibrios in this and similar solvents. Extraction by stirring of hot or cold alcoholic suspension of the vibrios, or refluxing in alcohol, was found to be successful but inefficient. Extraction of the dry vibrios in a Soxhlet apparatus was highly satisfactory. With methyl alcohol, ethyl alcohol, and chloroform the extraction proceeds rapidly, the bulk of the activity being extracted in 1-2 hours. It is not clear whether removal is quantitative in 24 hours; at that time large doses, *ca.* 30 mg (60 billion vibrios), are required to kill mice by intraperitoneal inoculation and such deaths are difficult to interpret. On the basis of mouse assay, however, all the original toxicity may be accounted for in the extract. There is appreciable destruction of the activity after 6-8 hours in the boiling solvent and the entire toxicity may be removed intact only by fractional extraction. Extraction with ethyl ether is considerably slower, 7/8 of the activity being removed in 72 hours.

The extracted material was yellowish in color and appeared to be a mixture of lipids, and in the case of alcoholic extracts contained considerable quantities of inorganic salts. Similar preparations may be made by precipitation of ground vibrios with alcohol, etc. In general, however, extraction of dried vibrios with alcohol or chloroform was most satisfactory. The toxicity was precipitated from alcoholic solution by acetone, the solution becoming opalescent with the addition of 2 volumes and a white flocculent precipitate is formed with 10 volumes. Attempts to develop a fractional acetone precipitation were not successful; the activity precipitated partially at various acetone concentrations. The toxicity could be purified by successive acetone precipitation and re-solution in minimal quantities of hot absolute alcohol and obtained as a white lipid material. The yellow color of the crude extract was associated with inactive lipid. The substance so prepared was negative to the Molisch, Million, and biuret tests in alcoholic solution, alcohol-water solution, or aqueous suspension. Minimal values of nitrogen and phosphorus found on analysis were 5% and 0.7% respectively. With proper attention to inactivation in the extraction process, it could be consistently prepared with a mouse MLD of 30 μg. It was precipitated from alcoholic solution by the addition of water, the solution becoming opalescent when a concentration of 20-25% alcohol was reached, but did not flocculate unless the alcoholic solution contained as much as 1% of the material. It was inactive in suspension in alcohol-water or -saline, but the activity

[9] Basu, C., Chaudhury, A., and Basu, R., *Calcutta Med. J.*, 1940, **36**, 571.

[10] Banerjee, D. N., *J. Ind. Med. Assn.*, 1942, **11**, 95.

was restored by re-solution in alcohol. This material represented about 2% of the dry weight of the vibrios.

This material was obviously impure since the solubilities of the activity varied somewhat according to the method of preparation. For example, after more than 3 successive acetone precipitations of an alcoholic extract, the material became increasingly difficult to redissolve in alcohol. Alcohol solutions of chloroform extracted material gave a flocculent precipitate with acetone, but a second precipitation gave only an opalescence. Similarly, material prepared by ether extraction is less soluble in alcohol-water than that extracted with alcohol, precipitating in 50% alcohol. Such variability is, of course, characteristic of an impure lipid. In this connection it may be noted that very small yields of highly active material could be obtained from later fractions in fractional extraction; in some instances a mouse MLD of 2 μg has been observed. It seems probable also that some of the nitrogen represented contamination.

In general, however, it is clear that the endotoxin of the cholera vibrio is (a) resistant to peptic or tryptic digestion, (b) stable to acid, (c) unstable to alkali (N/10 NaOH at room temperature), (d) readily soluble in methyl and ethyl alcohols, chloroform, and ether but not in glycols, (e) readily dialyzable, and (f) is closely associated, possibly identical, with a phospholipid. These and additional studies will be reported in detail in a later paper.

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The Endotoxin of the Cholera Vibrio: Immunological Properties.*

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The toxicity of the cholera vibrio seems to play an important role in the pathology of Asiatic cholera in man and the immune response to this toxicity is of some interest. The isolation and purification of the vibrio endotoxin reported in the preceding paper[1] has allowed a more precise investigation of this matter than has hitherto been possible. Certain aspects of these studies are herein reported in preliminary form.

There appears to be considerable confusion regarding the antigenicity of the cholera toxin since many workers have designated hemolytic strains as "toxic" and have worked with antihemolysins. Since it is now established that the "true" cholera vibrio is non-hemolytic,[2] such studies would seem to be irrelevant. Some workers, including Pottevin,[3] Horowitz,[4] Bail,[5] Hahn and Hirsch,[6] and Andu and van Niekerk[7] have reported the preparation of antisera which were protective *in vivo* against the lethal action of toxic extracts of the vibrios. It is generally admitted, however, that the

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

1 Burrows, W., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 306.

2 See, for example, the earlier studies of van Loghem, J. J., *Centralbl. f. Bakt., I Abt. Orig.*, 1926, **100**, 19; also Gardner, A. D., and Venkatraman, K. V., *J. Hyg.*, 1935, **35**, 262.

3 Pottevin, H., *Bull. Soc. Path. Exot.*, 1913, **6**, 409.

4 Horowitz, L., *Z. f. Immunitätsf.*, 1913, **19**, 44.

5 Bail, O., *Z. f. Immunitätsf.*, 1916, **25**, 248; *ibid.*, 1917, **26**, 97.

6 Hahn, M., and Hirsch, J., *Centralbl. f. Bakt., I Abt. Orig.*, 1927, **104**, 211; *Z. f. Hyg. u. Infektionskr.*, 1929, **110**, 355.

7 Andu, A. B., and van Niekerk, J., *Centralbl. f. Bakt., I Abt. Orig.*, 1929, **112**, 519.

antitoxic properties of antisera are only feeble at best. For example, Bail[8] attempted to account for the unaltered toxicity of serum-treated vibrios by the demonstration of free complement-fixing substances in immune guinea pigs infected with vibrios, these substances presumably representing uncombined toxin. According to Eisler and Kovacs[9] the toxin is not antigenic in that it is not specifically precipitated by antiserum, but adsorbed on the flocculating antigen-antibody complex.

In our experiments various preparations of endotoxin have been studied. The immunological activity of endotoxin prepared by preliminary extraction with alcohol and 3 subsequent precipitations with chilled acetone was indicated by skin reactions in immune rabbits and by specific precipitation and complement fixation with rabbit immune sera. The intradermal inoculation of 100 to 300 μg of endotoxin in 0.1 ml 25% alcoholic solution gave a skin reaction of the delayed type, appearing in 24 to 36 hours and fading appreciably in 48 hours, in immune rabbits but not in normal rabbits. An immediate toxic reaction, attributable only in small part to the alcohol and characterized by a circumscribed erythema followed by superficial necrosis, appeared in 1 to 3 hours and persisted for several days. It was not appreciably less in immune animals.

Specific precipitation could be demonstrated by the ring test and by a precipitation test of the Kahn type. In the former the antigen was used in solution in 25% alcohol and layered over the serum in the usual way; 25% alcohol gave a very slight clouding at the interface with both normal and immune sera which was not confusing. The precipitate appeared in 15 to 30 minutes at 37°C and disappeared almost entirely after 2 hours. Precipitation with H-O and O antisera occurred with antigen diluted to 1:5,000 to 1:10,000 as a rule though positive reactions were occasionally observed in dilutions as high as 1:400,000. With undiluted serum nonspecific reactions, *i.e.*, precipitation of the antigen with normal serum, were common and usually occurred to about ¼ the titer observed with immune sera. These could be reduced to 1:50 or less by 1:5 dilution of the serum; similar dilution of immune sera reduced the precipitin titer to 1:500 to 1:1,000.

Solutions of endotoxin preparations in absolute alcohol were used as antigens in the Kahn type of precipitation test. The antigen was found to be dispersed (cloudy but not granular) when mixed in the proportion of 0.01 ml antigen to 0.15 ml saline if the concentration of the endotoxin preparation was reduced to 0.72 mg per ml. This antigen was used both plain and cholestrinized (treated with 2 mg cholesterol per ml) and set up in 0.01 ml amounts with 0.15 ml of 1:5 and higher dilutions of inactivated serum. The cholestrinized antigen was found to be superior as indicated by more complete precipitation and often precipitation at higher serum dilutions. Readings were made immediately after shaking and were somewhat more clear-cut after standing 15 minutes. No precipitation was observed with normal rabbit serum. H-O and O antibacterial sera gave 3+ (flocculent precipitate without clear supernate) reactions in dilutions as high as 1:50 and 1:60, and a single antitoxic serum (see below) gave 4+ in 1:60 dilution.

The absolute alcoholic antigens were highly anticomplementary and the anticomplementary unit, approximately 0.35 mg, was unaffected by dilution in distilled water, 25% alcohol, physiological salt solution, and various concentrations of normal serum. Antigenicity disappeared almost entirely in distilled water dilution, the antigenic unit was approximately equal to the anticomplementary unit in 20% alcohol and serum dilution, and was about 4 times as large, *i.e.*, 0.09 mg, in saline dilution. The antigen was, therefore, used in saline dilution. Heating the diluted antigen at 56°C for 60 minutes did not affect its anticomplementary activity but reduced antigenicity by about half. Treatment with cholesterol increased both anticomplementary activity and antigenic activity, the former to 0.035 mg and the latter to 0.014 mg. In spite of the unfavorable alteration in the anticomplementary unit-antigenic unit ratio the cholestrinized antigen was more satisfactory

[8] Bail, O., *Z. f. Immunitätsf.*, 1916, **24**, 396.

[9] Eisler, M., and Kovacs, N., *Wien Klin. Woch.*, 1926, **39**, 469.

in the complement fixation reaction. In no instance did fixation occur with this antigen in the presence of normal rabbit serum. Only partial fixation, rarely 3+ and usually 2+, occurred with H-O, O and antitoxic sera, and in general 2+ fixation was observed in serum dilutions as high as 1:50. Antitoxic sera did not fix complement in higher dilution nor more completely than the antibacterial sera.

These observations appear to indicate at least a haptene-like function of the purified endotoxin preparations, though thus far the *in vitro* antigen-antibody reactions are relatively unsatisfactory for routine use.

In view of the low molecular weight indicated by its ready dialysis, it was not anticipated that endotoxin would stimulate antibody formation. Nevertheless, a series of rabbit immunizations was attempted with various types of preparations, including: (1) absolute alcohol solution of crude alcoholic extract; (2) suspension in 20% alcohol of alcoholic extract purified by 3 successive precipitations with chilled acetone (mouse MLD of final preparations was 35 to 40 μg); (3) preparations of dialysate of ground vibrios, precipitated after grinding with minimal amounts of trichloracetic acid, and dialyzed against daily changes of distilled water in the refrigerator for 14 days, (a) dialysate concentrated to contain 1 mouse MLD per ml, (b) dialysate evaporated to dryness, taken up in absolute alcohol, filtered and diluted to contain 20% alcohol (mouse MLD of final preparation 25 μg).

In the first experiments with alcoholic solutions only small amounts of material could be given because of toxicity; the lethal dose of endotoxin for the rabbit has not been determined accurately but appears to be possibly double that for the mouse, disregarding differences in body weight. A representative animal receiving a total of 42 μg of endotoxin in 3 intraperitoneal and 2 intravenous doses in a period of 14 days showed no agglutinin titer ($<$1:100) but in 0.1 ml amounts the serum protected mice against 100,000 MLD of mucin-suspended vibrios.† An additional intravenous inoculation of 3 mg was tolerated but demonstrable agglutinins did not appear. A second dose of 3 mg resulted in an agglutinin titer of 1:500 but there was no increase in protective titer. These, and similar results with other animals, were taken to suggest the independence of agglutinin and protective antibody.

As pointed out in the preceding paper,[1] suspensions of the alcoholic endotoxin in 20% alcohol in water or physiological saline were not toxic and larger amounts could be given. A number of preparations of acetone-precipitated material were used and the following experiment is representative. Following the intraperitoneal and intravenous inoculation of a total of 8.0 mg in 5 doses, the agglutinin titer was 1:1,000 and the serum protected passively immunized mice against 100,000 MLD of mucin-vibrios. Two additional intravenous inoculations of 1.6 mg each did not raise the agglutinin titer; in fact it fell to 1:500, but the protective titer rose to between 1,000,000 and 10,000,000. One animal in the series showed a very high agglutinin titer, 1:20,000, but similar titer of protective antibody.

The antisera to these alcoholic antigens have not been examined systematically for precipitin and complement-fixing antibody titer, but the few which were tested gave positive reactions.

In the experiments with dialysate it is to be emphasized that the barrier of dialysis through cellophane was interposed between the intact vibrio and the final preparation; consequently, no matter what the impurities in such preparations, large molecules that do not dialyze are not present. The animals immunized with the 2 types of dialysate preparations indicated above received a total of 7.5 to 8.5 mg in 5 or 6 doses, only the first of which was intraperitoneal. In all instances the protective titer was at least 100,000 and the agglutinin response marked, with all sera showing titers of 1:50,000 or more. As measured by agglutinin response, these antigens appear to be markedly superior to whole vibrios for a concentrated course of hyperimmunization with a total of 50 to 100 mg (100,000 to 200,000 million vibrios) has been

† Other aspects of this investigation, including detailed studies on protective antibody, are as yet unpublished.

found necessary to produce high agglutinin titers.‡

Mice could be actively immunized by the inoculation of alcohol-saline suspension of alcoholic toxin. In a typical experiment a total of 234 μg given in 3 intraperitoneal inoculations 4 days apart protected against 10,000 MLD of mucin-vibrios given 6 days after the last inoculation. In comparison with this, approximately the same degree of protection is afforded by the inoculation of not less than 1 mg of whole vibrios in heat-killed vaccine, and often a larger amount has been required.

On the other hand, it has not been possible to demonstrate an *in vitro* neutralization of the activity of endotoxin preparations by either antibacterial or anti-endotoxic sera known to be protective when tested against mucin-vibrios in the mouse. Thus, the toxicity of concentrated alcoholic solutions containing 1000 MLD per ml was not impaired by mixture with 2 volumes of antiserum having a protective titer of 100,000, the mixture being inoculated in 0.05 ml amounts either immediately or after incubation for as long as 6 hours. Furthermore, we have been unable to immunize mice, either actively or passively, against the lethal effect of intraperitoneal inoculation of the purified endotoxin. These titrations have all been carried out with dilution in powers of 10; it is possible that the more sensitive method of proportionate deaths would indicate some protection, but we have not used it because of its limited utility.

‡ Details of immunization procedure and agglutinin response will be reported elsewhere in a study on the antigenic structure of the cholera and related vibrios.

14793 P

The Endotoxin of the Cholera Vibrio: Action on Living Semipermeable Membranes.*

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The profuse diarrhea, a prominent feature of Asiatic cholera in man, is usually attributed to the action of the endotoxin of the vibrios; whether the pathology of organs such as the kidney is produced by absorbed toxin or is a consequence of the marked dehydration and hypochloremia is not clear. Though in general the pharmacological activity of the bacterial endotoxins is not characteristic, some workers have reported that intravenous inoculation of experimental animals with toxic extracts produces a symptom complex analogous to that of the early stages of human cholera. Hahn and Hirsch,[1] for example, reported that a profuse diarrhea with a loss of 10-17% of the body weight of fluids is produced in young rabbits and Ghosh[2] has made similar observations. Pham[3] has produced a diarrhea accompanied by marked albuminuria leading to emaciation and death in guinea pigs and rabbits; postmortem examination showed hemorrhagic infiltration of the terminal portion of the small intestine, congestion of Peyer's patches and desquamation of the mucosa together with some pathology of the kidney. Less characteristic findings have been reported by Sanarelli[4] and by Basu, Chaudhury, Basu.[5]

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

1 Hahn, M., and Hirsch, J., *Klin. Woch.*, 1928, **7**, 2483.

2 Ghosh, H., *C. R. Soc. Biol.*, 1933, **112**, 1176.

3 Pham, H. C., *C. R. Soc. Biol.*, 1935, **119**, 78.

4 Sanarelli, G., *Ann. Inst. Pasteur*, 1920, **34**, 370.

5 Basu, C., Chaudhury, A., and Basu, R., *Calcutta Med. J.*, 1940, **37**, 571.

In a few instances the effect of toxic extracts on isolated tissues, heart and intestine, has been studied. Perfusion of the heart with toxic extracts is reported to produce paralysis[6] and peristalsis of the intestine is said to be stimulated by small doses of toxin and inhibited by large doses.[1,6]

In our investigation of the pharmacological activity of purified endotoxin isolated from the cholera vibrio,[7] the effect of the toxin on the permeability of living membranes to fluid has been of some interest. Frog skin from the ventral surface and small intestine of the guinea pig and rabbit have been used; other membranes such as rabbit omentum proved too fragile or otherwise unsatisfactory. Graduated columns were made from serological pipettes, usually of 5 ml capacity. The tops were cut off and flanged slightly to facilitate filling. Thistle tubes, 30 mm in diameter, were sealed on in place of the tips for the experiments with frog skin and the skin gently stretched over the opening and tied on. Short lengths of glass tubing 3 mm in diameter for guinea pig intestine and 5 mm for rabbit intestine, were sealed on in place of the tips of other pipettes. Pieces of intestine, approximately 6 cm in length, were taken from the freshly killed animal, one end slipped over the end of the graduated column and tied on and the other end tied off. The upright column was then filled with Ringer-Locke solution and the lower end and membrane immersed in the same solution in a small beaker and incubated in a water bath. The hydrostatic pressure of 8-10 inches on one side of the membrane was sufficient to produce an appreciable rate of flow across the membrane which was measured by periodic observation of the volume in the column. Measurement of the volume within the membrane and column at the beginning and end of such experiments indicated that distention of the membrane was not quantitatively important. The rate of flow was approximately linear with respect to time except for a latent period in the first 30-60 minutes (See Fig. 1). In preliminary experiments the addition of 20% sucrose to the solution in the beaker did not increase the rate of flow and was appreciably toxic to the membranes.

Skin from the ventral surface of the frog was considerably more satisfactory than the dorsal skin but the direction of flow through the skin was not important. In the case of small intestine there appeared to be no significant differences between duodenum, jejunum, and ileum. Here also the direction of flow was not material, there being no advantage and considerable disadvantage in turning the piece inside out. Peristalsis persisted for 8-10 hours at 37°C and for 18 hours or more at 24°C but no experiment with intestine was carried longer than 6-7 hours. The rate of flow did not differ appreciably over this temperature range. Experiments with frog skin were carried out at 24°C for as long as 12 hours. In view of the fact that with strips of intestine the flow was not from the lumen to the circulation but through the musculature as well, it was of interest that the rate was almost precisely the same as through frog skin. In general, however, frog skin was most satisfactory though of limited utility for our purposes, guinea pig intestine less so and rabbit intestine least satisfactory with respect to reproducibility of results. In part because of unavoidable differences in size of the pieces of intestine, there was considerable variability from one piece to another in rate of flow. When experiments were run in quadruplicate, however, results were closely reproducible.

The rate of flow of fluid through the normal membrane immersed in Ringer-Locke solution was found to be about 0.25 ml per hour. The addition of living vibrios, or crude or purified toxin to the solution either within or outside the membrane markedly accelerated this rate. Purified toxin was added as a concentrated alcoholic solution, the toxin precipitating to give an opalescent suspension in the Ringer-Locke solution; control experiments indicated that the small amount of alcohol added was not important. The acceleration of flow began to be apparent at a concentration of 0.5 MLD (Mouse) per ml and increased with increased concentration of toxin until with 4 MLD per

[6] *Cf.* Andu, A. B., and van Niekerk, J., *Centralbl. f. Bakt., I Abt. Orig.*, 1929, **112**, 519.

[7] Burrows, W., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 306.

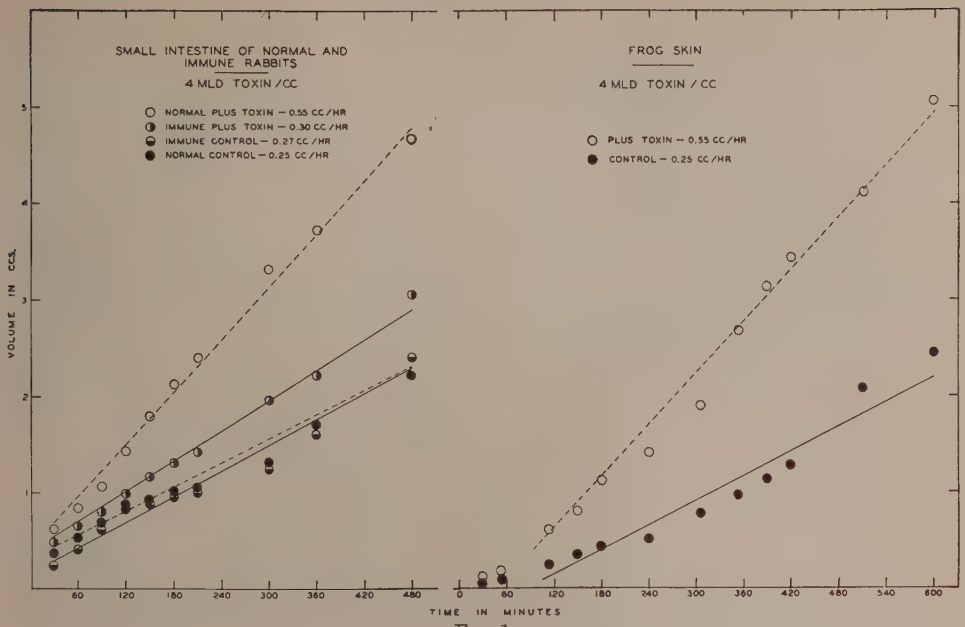

FIG. 1.

The effect of the endotoxin of the cholera vibrio on the permeability of living membranes to fluids. Left, isolated strips of intestine from normal and immune rabbits. Note that the toxin approximately doubles the rate of flow of fluid in the normal intestine, while the intestine from the immune animal does not differ significantly from the controls. Right, frog skin. Note the precise duplication of results with the normal rabbit intestine. Rates were determined graphically and lines fitted by inspection.

ml the rate was approximately double that of the controls as shown in Fig. 1. At this concentration the toxin appeared to have no deleterious effect on the membrane, and did not influence peristalsis of the strips of intestine. With higher concentrations more rapid flow could be produced—we have observed as much as 6-fold differences—but the results were inconsistent and erratic. It was immaterial whether the toxin was placed inside or outside the membrane, within or outside the lumen in the case of strips of intestine, and for the most part it was put inside because of the smaller volume and consequent economy of toxin. When crude toxin, such as ground vibrios, or vibrios precipitated with minimal amounts of trichloracetic acid, was used, it could not be detected in the outside solution at the end of the experiment by inoculation of mice with pooled and concentrated solution. Purified toxin, however, diffused through the intestine and assay by mouse inoculation indicated that by the end of the experiment the concentrations inside and outside were approximately the same. The acceleration of flow occurred only in the presence of toxin for strips of intestine soaked over night in Ringer-Locke solution containing 4 MLD of toxin per ml and thoroughly washed before use did not differ from normal controls.

When the increased permeability was produced by suspensions of living vibrios, the addition of antiserum agglutinated the bacteria and the accelerating effect was no longer apparent. The effect of endotoxin, either crude or purified, however, could not be neutralized by antiserum, even hyperimmune serum showing agglutinin titers of 1:50,000 and powerful protective properties by mouse assay, in concentrations as high as 10% in the toxin-Ringer-Locke solution. Nor were strips of intestine soaked in such immune serum solutions overnight appreciably resistant to the effect of the toxin. If, however, strips of intestine were taken from immune animals they were completely, or almost com-

pletely, resistant to the action of the toxin and in its presence showed little or no difference in permeability to fluids from normal intestine in the absence of toxin. Data from typical experiments are given in graphical form in Fig. 1.

These phenomena have been studied in detail and the investigation extended considerably further than indicated here; these studies will be reported fully elsewhere. It may be pointed out, however, that these results are consistent with and complementary to those reported in a preceding paper[8] and it is apparent that active immunity to Asiatic cholera in the experimental animal, and presumably also in man, includes antitoxic as well as antibacterial immunity. The significance of the former in enteric infection with the vibrios is clearly indicated though not as yet quantitatively evaluated. If it be assumed that the resistance of the immune intestine to the action of the endotoxin has an antigen-antibody basis, and if this is not assumed an entirely new type of immunity must be postulated, it is clear that the functional antibody cannot be circulating antibody in the isolated strip of intestine and may, therefore, be intracellular antibody, presumably in the macrophages of the tissues of the intestine.

[8] Burrows, W., Mather, A. N., Wagner, S. M., and McGann, V. G., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 308.

14794

Influence of Citric Acid on Blood Pyruvate Levels in Man.

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Von Euler and Högberg[1] reported that the administration of citric acid produces a delayed decrease in the concentration of pyruvic acid in the blood. The principal evidence was from a small series of experiments with rats receiving oral or subcutaneous dosages of sodium citrate. Emphasis, however, was placed on a single experiment with a normal man whose blood pyruvate was lowered to 22% of the original level 12 to 15 hours after the oral ingestion of 2 g of citric acid as the sodium salt. The blood pyruvate change in this man was stated to be accompanied by indications of a profound general disturbance including sweating, shivering, and fever.

Apart from the suggestion of important theoretical relations this report is of interest because of the growing use of blood pyruvate measurements in the study of thiamine deficiency and the fact that the citric acid dosage employed is scarcely heroic. Two grams of citric acid would be supplied by one large lemon or by 200 cc of orange juice.[2]

In this Laboratory this question was studied in 5 experiments on 3 normal young men who had been maintained on a carefully standardized dietary for several months. In each experiment a blood sample was drawn before breakfast at 8:00 a.m. and 3 g of citric acid as Na citrate were administered orally in 200 cc of orange juice on the same day at 6.00 p.m. The final blood sample was taken before breakfast on the following morning at 8:00 a.m., that is, 14 hours after the citric acid administration. The blood samples were drawn from veins in the antecubital fossa. Pyruvate was estimated in duplicate by the method of Friedemann and Haugen.[3]

The results are summarized in Table I. It is clear that the citric acid had no effect of any kind on the pyruvate level at the time—14 hours—when the maximum change was

[1] Von Euler, H., and Högberg, B., *Z. Physiol. Chem.*, 1940, **265**, 244.

[2] Chatfield, C., and Adams, G., *U. S. Dept. Agric. Circular* No. 549, 1940.

[3] Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, 1943, **147**, 415.

TABLE I.
Blood Pyruvate Values in mg per 100 cc of Whole Blood Before and 14 Hours After the Ingestion of 3 g of Citric Acid.

Time	Subject 1			Subject 2	Subject 3
1. Before citric acid	0.56	0.96	1.06	0.58	0.73
2. After " "	0.58	0.70	1.03	0.72	0.67
△, 1-2	—0.02	+0.26	+0.03	—0.14	+0.06

expected from the report of von Euler and Högberg. Clinical effects and subjective complaints were likewise entirely absent in all of the present experiments. Blood lactate, which was also measured in 2 experiments, did not change. We are unable to offer any explanation for the divergence of these results from those previously reported but we may note that the details in the von Euler and Högberg report are scanty.

14795 P

Evacuation of Gall Bladder in Female Patients with Pernicious Anemia.

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In an earlier study[1] it was observed that in male patients with pernicious anemia the rate of evacuation of the gall bladder was not significantly retarded by the disease, but that in a surprisingly large proportion of the cases studied roentgenologically, the gall bladder could not be visualized, notwithstanding the use of the intravenous method and the absence of any gall bladder history. Accordingly it seemed desirable to examine a comparable number of female patients.

The present report is based upon cholecystographic studies of 48 consecutive, unselected patients with pernicious anemia (23 males and 25 females). In this group, 42% could not be visualized (35% of the males and 48% of the females). Also, a review of the 31,311 necropsies recorded by the Department of Pathology for the 15-year period between 1926 and 1940, showed that of the 105 individuals having pernicious anemia, 32.4% had had either cholecystitis or cholelithiasis (or both) or had had the gall bladder removed. Furthermore the incidence of gall bladder disease increased progressively with age to the 8th decade—whereas Blalock[2] has shown that the greatest percentage of cases of biliary disease occurs in the fifth decade. These figures suggest that pernicious anemia increases the incidence of gall bladder disease and may have an etiological relation to it.

Evacuation of the gall bladder in 12 female patients (average age, 58 years) showed marked retardation over the group of female controls[3] (average age, 65 years); for in the first 40 minutes after the standard meal, the pernicious anemia group had evacuated only 71.5% of the contents of the gall bladder as against 84% by the controls. The difference is statistically significant, being nearly 3 times the standard error.

It would thus appear that patients with pernicious anemia are more likely to have developed cholecystic disease than other individuals of comparable age. When one asks what changes occur in this disease which might affect the gall bladder, the factor of increased excretion of fecal urobilinogen imme-

[1] Layne, J. A., and Boyden, E. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 534.

[2] Blalock, Alfred, *Bull. Johns Hopkins Hosp.*, 1924, **35**, 391.

[3] Boyden, E. A., and Grantham, S. A., Jr., *Surg., Gyn., and Obstet.*, 1936, **62**, 34.

diately comes to mind. In patients in a state of relapse it may be increased several times its normal value and following liver therapy it sharply decreases.[4] It is conceivable, therefore, that excessive secretion of bile pigments into the biliary tract may have produced temporary obstruction of the cystic duct with consequent damage to the gall bladder wall.

The greater damage sustained by the female gall bladder, together with the slower rate of emptying in patients of this sex, may be explained on the basis of the greater susceptibility to gall bladder disease of women who have borne children,[5] for in all but 2 of the group the gall bladder had been subjected to the double insult of the stasis of pregnancy and that of pernicious anemia.

[4] Watson, C. J., *Arch. Int. Med.*, 1931, **47**, 698.

[5] *Cf.* Gerdes, M. M., and Boyden, E. A., *Surg., Gyn., and Obstet.*, 1938, **66**, 145.

14796

Experimental Production of Negative Pressure in the Frontal Sinus.

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The clinical syndrome known as vacuum headache has long been recognized but there seems to have been no demonstration of a reduced pressure in the sinuses or of a mechanism capable of producing it. When one of us (Hilding[1]) demonstrated that negative pressure can be produced by normal ciliary action in the lower part of the respiratory tract, it was decided to determine if a similar mechanism would produce negative pressure in paranasal sinuses.

Technic. With the animal under ether anesthesia and with observance of aseptic technic two 16-guage hypodermic needles were inserted into one frontal sinus of each dog. The needles were introduced through small holes made through the scalp and bone. Each needle fitted into both the skin and the bone effectively enough to be airtight. One needle was connected to a water manometer and the other to a syringe containing the mucinous secretion to be injected. A third needle, introduced into the opposite frontal sinus and connected to another water manometer, acted as control.

The mucinous secretion was collected from the trachea of either the dogs used in these experiments or other dogs.

[1] Hilding, A. C., *Ann. Otol., Rhin., and Laryng.*, 1943, **52**, 816.

Experiment 1. A few cubic centimeters of mucus was injected into the left frontal sinus of animal 1 at 2:15 p.m. The first portion entered without resistance but soon a resistance was felt, which increased to +270 mm of water when the injection was finished and the needle closed. In two minutes the pressure had fallen to +83 mm and it passed zero and became negative between the fifth and the sixth minute. Twenty minutes later the manometer reading stood at —50 mm and it reached a maximal negativity of —59.5 mm forty-seven minutes after injection. It remained stationary for about 7 minutes and then rose to about —45 to —48 mm where it remained until 2 hours and 6 minutes after injection, when the experiment was terminated.

Experiment 2. At 9:51 a.m., 3.5 cc of mucus was injected into the left frontal sinus of animal 2. At 10:07 a.m. the manometer recorded —3 mm of water. It varied between —3 and +8 mm until 11:00 a.m. when the experiment was concluded and the sinus opened. The mucus was about gone. The ostium was unusually large and, although mucus was passing through, the ostium was not filled or occluded by the mucus.

Experiment 3. Seven cubic centimeters of foamy mucus was injected into the left frontal sinus of animal 3 at 11:06 a.m. The imme-

diate pressure recorded on the manometer was +15 mm of water. The pressure declined to zero in 5 minutes and then became negative. The negative pressure increased rapidly for 12 minutes to reach —58 mm and then more slowly for another 12 minutes to reach a maximal negativity of —66 mm. The reading remained almost stationary for 5 minutes and then changed slowly to —32 mm, where it stood when the experiment was concluded after 4 hours and 39 minutes. Blockage of the needle with blood and mucus apparently had prevented return of the pressure to zero.

Experiment 4. Five cubic centimeters of foamy mucus was injected into the left frontal sinus of animal 4 at 11:18 a.m. The manometer reading stood at zero at 11:20. Negative pressure then began to develop and increased steadily to a maximum of —38 mm of water in 18 minutes. It varied between —36 and —38 mm for 7 minutes and then changed to —34 where it stood at 11:49. The animal was decapitated at 11:50 and the needles were removed and cleaned.

The needles were reinserted and 6 cc of mucus was injected at 11:59. Immediately after injection, the manometer reading stood at zero. Negative pressure began to develop after a minute or two and reached a negativity of —16 mm thirteen minutes after injection and 22 minutes after decapitation of the animal. The readings varied during the next 20 minutes between —8 and —22 mm. When the experiment was concluded at 12:32 p.m., the pressure was —16 mm.

Experiment 5. At 10:21 a.m., 6 cc of foamy mucus was injected into the right frontal sinus of animal 5. A slight positive pressure (+10 mm of water) was recorded on the manometer immediately but it passed the zero mark and became negative during the second minute and at 10:23 the reading was —6 mm. The negative pressure increased to —28 mm during the next 6 minutes. Then a leak seemed to develop about the needle and a larger sized needle was substituted. At 10:35 the pressure was again —7 mm and negativity progressively developed to reach a maximal negativity of —36 mm 19 minutes later (10:54 a.m.).

The femoral artery was cannulated and exsanguination begun at 11:05. When respirations ceased 3 minutes later the pressure stood at —30 mm and at 11:16 at —25 mm.

The animal was decapitated at 11:18 without disturbing the needles. At 11:19 the pressure was —22 mm; then for some unknown reason the change of pressure reversed and negativity increased again to —34 mm 19 minutes after respirations had ceased (11:27). The pressure varied between —24 and —34 mm until the experiment was terminated at 11:50, 42 minutes after death of the animal and 32 minutes after decapitation.

Meanwhile 7 cc of mucus was injected into the left frontal sinus of the same head and the sinus connected with a second manometer at 11:24, six minutes after the animal had been decapitated. A pressure of +4 mm was indicated one minute later and 3 minutes later the reading was —1 mm. An increasing negative pressure developed until, 20 minutes after injection, it amounted to —43 mm. When the experiment was terminated, 26 minutes after injection of mucus (11:50), the pressure was —41 mm (Fig. 1).

Comment. A marked negative pressure was produced in 5 of 6 normal sinuses by the injection of sufficient mucus to occlude the ostium. The failure to produce negative pressure in the second experiment probably resulted from the unusually large ostium found in that sinus.

The negative pressure found in the other 5 was presumably caused by the removal of mucus through the ostium by ciliary power. While the ostium remained occluded by mucus, air could not enter to replace the mucus as the latter was removed. It seemed improbable after the first 3 experiments that another mechanism such as absorption of air from the sinuses could have caused negative pressures of such magnitude.

Experiments 4 and 5 were designed to determine with certainty whether or not the air could have been removed by absorption through the blood stream. Hence both of these animals were decapitated and the experiment was repeated on the detached heads. In the case of animal 4, the experiment was repeated in the same sinus and in the case of animal 5 in the opposite sinus. In both

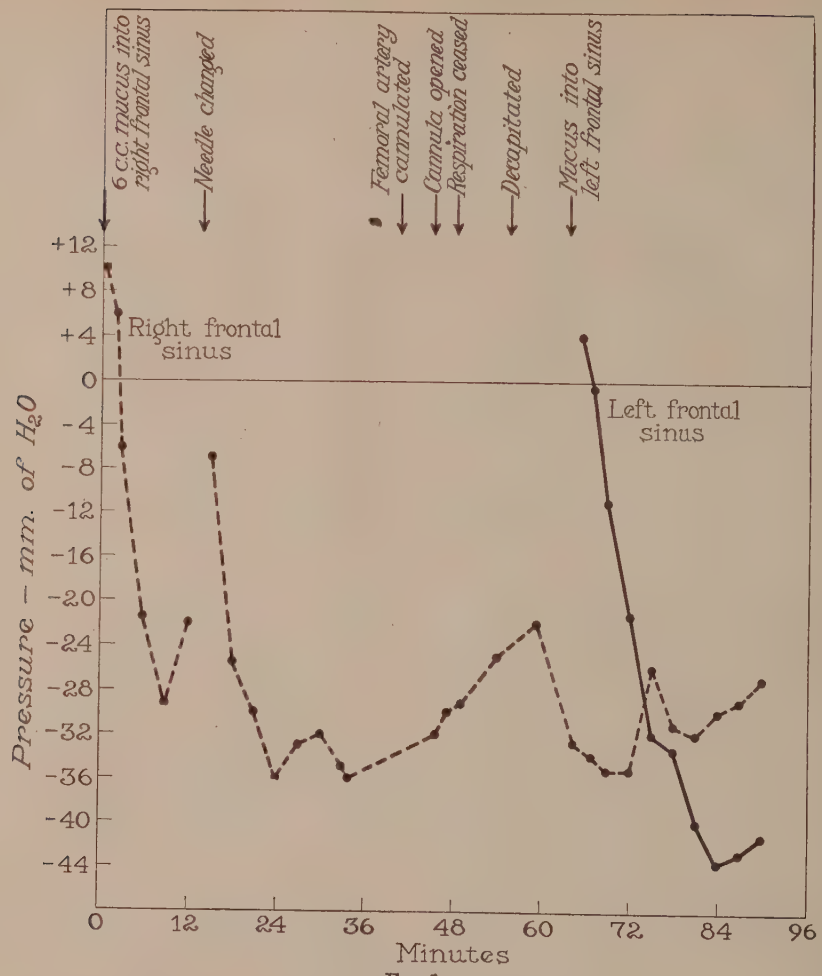

FIG. 1.

Negative pressure developed both before and after decapitation of the animal in experiment 5. Mucus was injected into the right frontal sinus and the development of negative pressure observed and recorded (dotted line above). The animal was then bled to death and decapitated without disconnecting the needles or manometer. There was some variation, as indicated. Mucus was injected into the left frontal sinus also but not until after decapitation. A greater negative pressure developed in the left sinus than in the right (solid line).

instances, there resulted negative pressure comparable to that which occurred before decapitation. In animal 4 the negative pressure after decapitation was somewhat less than before decapitation and in animal 5 somewhat greater.

Conclusions. 1. Negative pressure can be produced by ciliary activity within a normal sinus after a considerable quantity of mucinous secretion (from the same species of animal) has been injected. 2. This negative pressure occurs even after death and decapitation of the animal and is therefore not due to absorption of air by the blood stream.

14797

Factors Affecting the Reaction of Mice to Low Atmospheric Pressures.*

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In the course of work reported previously[1] we observed that mice died immediately after exposure to a sudden reduction of atmospheric pressure to 70 mm. Those that had previously been allowed access to a mixed food showed a marked congestion of the lungs, causing the surface to appear very dark, almost black. Those that had been deprived of food for approximately 24 hours, although they died just as promptly, did not show this darkening or blackening of the lungs.

In the present paper we wish to report on the effect of administering amino acids, protein, fat, carbohydrate, and sodium chloride, separately, upon the incidence of such pulmonary congestion. We have also conducted some experiments in which the mice were allowed to breathe oxygen, oxygen plus 5% carbon dioxide and air plus 5% carbon dioxide for a short time before the exposure to low pressure, and now report upon the effect of various foods upon the proportion of the animals surviving such treatment.

Experimental. White mice of 14 to 20 g were fed a standard commercial food (Fox Food Blox) and were housed in metal cages containing sawdust. Animals of different sexes were kept separately. At 2 p.m. of the day before the tests, mice which had been provided with adequate food and water since the previous day were transferred to cages with wire screen bottoms and subsequently allowed access to water but not to food. These animals were divided into two groups. Some were tested at various times between 20 and 27 hours after being deprived of food, and were found not to develop the characteristic lesions of the lungs. Other animals were either allowed continuous access to food (nonfasting), or, after the fast, were given access to food (fasting plus food) or had the various substances that were to be tested administered, either intragastrically or intraperitoneally as indicated in the tables, before being exposed to low pressure.

The apparatus used and the method of using it were the same as those employed in our previous work. As in the earlier paper, we have designated as "injured lungs" those showing congestion in at least half the surface, and as "completely injured" those in which the entire surface was congested.

By a slight change in the apparatus, various gas mixtures could be circulated through the desiccator containing the mice, for 10 minutes before reducing the pressure. Because the desiccator that we used for these experiments was larger than the one previously used, a pressure of 16 mm of mercury was necessary in the reservoir tanks, and about 30 seconds were required for the entire system to come to 70 mm.

In these experiments with high oxygen tensions, we were careful to use mice which were fairly uniform in weight, since, apart from other factors, smaller animals seemed to show a somewhat higher survival rate. This is in agreement with the experience of other workers.[2] We tested about 90 fasting mice and about 50 mice after feeding for one or 2 hours on the mixed food subsequent to the fast. A smaller number of mice were tested one or 2 hours after the administration of 0.6 cc of olive oil or one hour after the administration of 0.6 cc of 10% glucose. The oil was given by stomach tube, while the glucose was administered either by stomach tube or by intraperitoneal injection. Since the results which were obtained by both methods of administration of glucose were the same, they are combined in Table I.

The sudden fall in pressure did not seem to

* This research was supported by the California Fruit Growers Exchange Research Fund of the College of Medicine, New York University.

1 Kibrick, A. C., and Goldfarb, A. E., in press.

2 Van Liere, E. J., *Anoxia, Its Effects on the Body*, Chicago, 1942, p. 213.

TABLE I.

	No. of animals	Survivals, %	Dead, No.	With injured lungs, % of No. dead	With compl. injured lungs, % of No. dead
		Oxygen.			
Fasting	90	50	45	40	11
" + food	55	2	54	100	86
" + 10% glucose	12	8	11	90	82
" + olive oil	12	83	2	50	50
Non-fasting	28	18	23	91	61
		Oxygen + 5% CO_2.			
Fasting	49	53	23	26	4
" + food	46	7	43	98	56
		Air + 5% CO_2.			
"	24	0	24	8	0
" + food	18	0	18	72	44
		Without Previous Ventilation.			
"	48	0	48	12	4
" + food	44	0	44	96	67

excite the animals, and an interval of several seconds at 70 mm was necessary before they showed signs of discomfort. Some of the surviving mice were killed with ether, and their viscera were examined. Although a few of the animals were found to have developed lesions of the lungs, most of them had no obvious injury in any of their viscera. Once past the day after the tests, the surviving mice gained weight like normal animals and were susceptible in the usual way when again subjected to the low pressure. While we can not be certain, the absence of any apparent injury in the survivors which had received the oxygen would seem to indicate that the fatalities were due to acute anoxia.

That the greater number of survivals in fasting mice previously supplied with oxygen-rich mixtures was not due to the longer time required for the attainment of the low pressure in the larger chamber which was used in these experiments is shown by the figures in the lower part of Table I. Nor, apparently, did this longer interval of time affect the relative number of animals showing lesions in the lungs.

Results. Whereas fewer than 1% of the mice which previously respired in atmospheric air or in air plus 5% carbon dioxide survived exposure to 70 mm,[1] more than half of the fasting mice which previously respired in oxygen or in oxygen plus 5% carbon dioxide for 10 minutes were not killed by this treatment. Campbell[3] found a slightly greater survival rate in fasting rats subjected to diminished pressure for periods of about 30 minutes than in rats which had not been deprived of food. However, his results with fasting rats did not show the marked differences which he found with rats which had been fed carrots exclusively for several days. This is contrary to our own experience with mice exposed to a pressure of 70 mm after previous treatment with oxygen. We have been unable to detect any marked differences between the susceptibility of mice which had been fed exclusively on carrots for 5 to 7 days and that of fasting mice.[4]

This greater resistance to the effects of low pressure was nearly completely abolished by the taking of mixed food or by the administration of glucose. In marked contrast, the proportion of survivors after the administration of olive oil was even greater than among the fasting animals.

In Table II, we present data showing the effect of the administration of various substances upon the incidence of pulmonary congestion in mice exposed to a pressure of 70 mm without previous treatment with oxygen. Slightly different results were obtained with the 2 series of mice that were allowed access to mixed food after the fast. They are, there-

[3] Campbell, J. A., *Quart. J. Exp. Physiol.*, 1938, **28**, 231; 1939, **29**, 259.

[4] Kibrick, A. C., unpublished.

TABLE II.

Dose				Time elapsing between the administration of the substance and the exposure to 70 mm pressure									
			Minutes	0	5	15	30	45	60	90	120	180	240	300
Material	Vol., cc	Route*		No. of animals										
10% egg albumin	0.6	G	Total			11	6	12	18	12		12	10	
			With injured lungs			2	0	1	0	1		0	2	
			Compl. injured lungs			1	0	1	0	1		0	1	
Olive oil	0.6	G	Total						6		6	11	17	
			With injured lungs						0		0	2	1	
			Compl. injured lungs						0		0	0	0	
10% xylose	0.6	G	Total			6	6		6		6	5	6	
			With injured lungs			0	1		2		1	0	0	
			Compl. injured lungs			0	1		0		1	0	0	
10% alanine	0.6	G	Total			11	6		5	6	6	6	6	
			With injured lungs			8	1		3	3	3	5	2	
			Compl. injured lungs			1	0		1	1	1	3	2	
10% leucine	0.6	G	Total			6	6		9		6	6	12	
			With injured lungs			3	1		2		1	2	1	
			Compl. injured lungs			3	1		0		0	2	0	
0.9% NaCl	0.6	G	Total			6	6		6		6	6	12	
			With injured lungs			0	0		0		1	0	3	
			Compl. injured lungs			0	0		0		0	0	0	
3% NaCl	0.6	G	Total			12	6		6	6	6	12	12	
			With injured lungs			0	0		0	1	3	0	1	
			Compl. injured lungs			0	0		0	1	1	0	1	
0.9% NaCl	0.6	P	Total				6		6		5	6		
			With injured lungs				0		0		0	0		
			Compl. injured lungs				0		0		0	0		
1 or 2 hr feeding of Fox Food Blox			Total	24			10		18		12	12		
			With injured lungs	24			10		18		12	4		
			Compl. injured lungs	20			7		16		11	2		
1 or 2 hr feeding of Fox Food Blox			Total	24			10		18		12	18	17	
			With injured lungs	24			10		18		12	16	9	
			Compl. injured lungs	21			9		15		10	13	7	
10% glucose	0.6	G	Total		6	12	6	6	19		8	8		
			With injured lungs		3	8	6	4	14		7	3		
			Compl. injured lungs		0	6	5	2	9		6	0		
" "	0.6	G	Total				6		12	6	12	16	6	5
			With injured lungs				6		12	4	8	12	6	1
			Compl. injured lungs				5		10	2	5	8	3	0
" "	0.6	P	Total			6	12		11	12	11	6	6	
			With injured lungs			3	10		4	9	11	5	4	
			Compl. injured lungs			0	7		1	7	4	3	3	
10% starch	0.6	G	Total		6	18	18	6	18	6				
			With injured lungs		2	10	12	4	9	0				
			Compl. injured lungs		2	6	4	2	2	0				
" "	0.6	G	Total			6	6		6	6	6	6	6	6
			With injured lungs			2	5		6	5	5	6	4	2
			Compl. injured lungs			1	3		2	3	1	2	0	1
				Hours of fast										
				20		21		22		24		26		27
				Number of animals										
None			Total	36		18		36		36		25		18
			With injured lungs	8		1		4		4		4		2
			Compl. injured lungs	3		1		2		1		1		1

* G = intragastric. P = intraperitoneal.

fore, presented separately. Somewhat different results were also obtained with the 2 series of mice which received glucose or starch by stomach tube.

As may be seen from the table, only starch and glucose acted as did the mixed food in increasing the proportion of mice showing injured lungs, and glucose had this effect whether it was given intragastrically or intraperitoneally. Just as in the mice that were previously treated with oxygen, the action of olive oil seemed to protect against injury, rather than sensitize to it.

Summary. Mice that had been permitted access to a mixed food before exposure to high tensions of oxygen with a subsequent sudden fall of total pressure to 70 mm were found much more likely to succumb to such treatment than were mice that had been deprived of food for a day. This effect of food was also observed after the administration of glucose, intragastrically or intraperitoneally, but not after the intragastric administration of olive oil.

Mice not previously treated with high tensions of oxygen were nearly all killed by the sudden exposure to 70 mm pressure, but those that had been deprived of food did not show the marked congestion of the lungs that was observed in mice that had been fed a mixed food. Mice that had received glucose or starch showed this congestion, but those that had received xylose, olive oil, egg albumin, leucine, or sodium chloride did not.

14798

Studies on Rates of Hydrolysis of N^4 Dibasic Acid Substituted Sulfonamides.*

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The reports of Poth, Knotts, Lee and Inui[1] and Poth and Ross[2] detailed the results of a study of 33 sulfonamides which were examined for bacteriostatic activity in the gastrointestinal tract. Fourteen of these compounds were N^4 acyl (dibasic acid) derivatives. Succinylsulfathiazole and phthalylsulfathiazole, of this series, have been shown to possess considerable local bacteriostasis in the gastrointestinal tract and were studied extensively both experimentally and clinically.[3-11]

Many of the other members of this series were found to have little antibacterial activity in the bowel. Since the conjugated sulfonamides have but slight bacteriostatic activity, the simplest explanation of the activity of these compounds should be due to the free sulfonamides liberated by the hydrolysis of the acylated compounds. If this were true, then the rates of hydrolysis of these sulfonamides in aqueous solutions of varying hydrogen ion concentrations should furnish an index to their antibacterial activity.

Poth, Knotts, Lee, and Inui[1] observed that

* Supported by a grant from Sharp & Dohme, Inc., Philadelphia, Penn.

1 Poth, E. J., Knotts, F. L., Lee, J. T., and Inui, F., *Arch. Surg.*, 1942, **44**, 187.

2 Poth, E. J., and Ross, C. A., *Tex. Rep. Biol. Med.*, 1943, **1**, 345.

3 Poth, E. J., Chenoweth, B. M., Jr., and Knotts, F. L., *J. Lab. and Clin. Med.*, 1942, **28**, 162.

4 Smyth, C. J., Gould, S. E., and Finkelstein, M. B., *J. A. M. A.*, 1943, **121**, 1244.

5 Hardy, A. V., Burns, W., and DeCapito, T., *Pub. Health Rep.*, 1943, **58**, 689.

6 Twyman, A. H., and Horton, G. R., *J. A. M. A.*, 1943, **123**, 138.

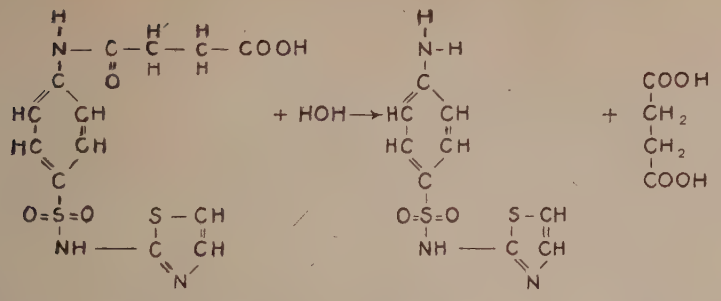
7 Poth, E. J., *J. A. M. A.*, 1942, **120**, 265.

8 Behrend, M., *S. Clin. North America*, 1944, **24**, 238.

9 Crohn, B. B., *Gastroenterology*, 1943, **1**, 140.

10 Bloomfield, A. L., and Lew, W., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 28.

11 Menchaca, F., *An. Soc. puericult.*, Buenos Aires, 1942, **8**, 399.



Succinylsulfathiazole *Sulfathiazole* *Succinic Acid*

those compounds which were easily hydrolyzed to the free aryl amine, were readily absorbed, and were usually more toxic than the relatively stable derivatives. It was observed by Poth and Ross[12] that a much higher concentration of free sulfathiazole was obtained in feces with phthalyl derivative of sulfathiazole than with the succinyl derivative when equal doses of the drugs were administered. This observation may be taken to explain why phthalylsulfathiazole is twice as effective bacteriostatically as succinylsulfathiazole. While the concentration of free sulfonamide in the feces may not account for the total bacteriostatic effect, it would seem that it may be related in some manner to the activity of members of this series of compounds.

Method. Millimolar solutions of the N^4 acyl sulfonamides are made in 1.0 N-HCl and 1.0 N-NaOH and maintained at 38°C. Samples are withdrawn at the desired time intervals and quantitative estimations of the concentration of the free aryl amine made using a modification of the colorimetric procedure of Bratton and Marshall.[13] An Evelyn colorimeter is employed.

Rates of Hydrolysis of the Conjugated Sulfonamides. When the conjugated compounds in solution are treated with acid or alkali, the compounds are split to yield a molecule of the free aryl amine, or free sulfonamide, and a dibasic acid: *i.e.*,

If the N^4 substituent is a saturated dibasic organic acid, the hydrolysis rates are more rapid in alkaline than in acid solution as shown in Charts I and II and Table I-A. First order equations do not fit these hydrolysis curves. If, however, the N^4 substituent is the unsaturated organic acid, maleic acid, then the speeds of the hydrolysis rates in alkali and acid are reversed (See Table I-B). Likewise, if the N^4 substituents are the aromatic organic acids, phthalic and quinolinic acids, the rates

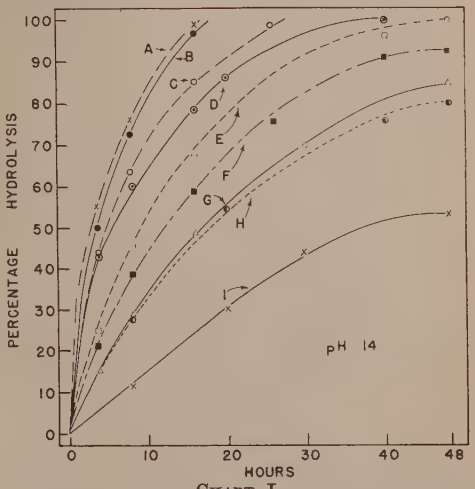


CHART I.

Graphic representation of the rates of hydrolysis of N^4 aliphatic dibasic acid substituted sulfonamides in 1.0 N-NaOH at 38°C.

A. Succinylsulfaguanidine
B. Oxalylsulfathiazole
C. Succinylsulfapyridine
D. Succinylsulfathiazole
E. Succinylsulfanilamide
F. Maleylsulfathiazole
G. Malonylsulfathiazole
H. Succinylsulfamethyldiazine
I. Maleylsulfanilamide

Each compound is present in one millimolar concentration.

[12] Poth, E. J., and Ross, C. A., *J. Lab. and Clin. Med.*, 1944, **29**, 785.

[13] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **125**, 537.

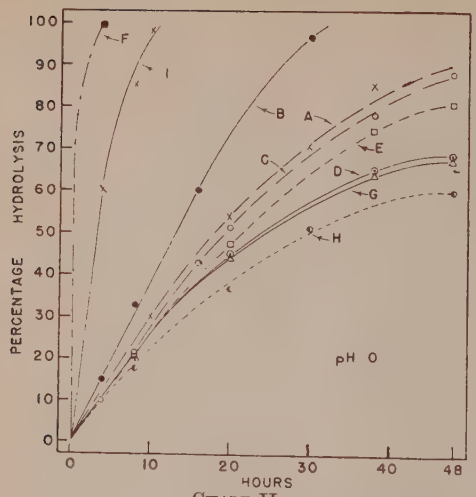


CHART II.

Graphic representation of the rates of hydrolysis of N^4 aliphatic dibasic acid substituted sulfonamides in 1.0 N-HCl at 38°C.

A. Succinylsulfaguanidine
B. Oxalylsulfathiazole
C. Succinylsulfapyridine
D. Succinylsulfathiazole
E. Succinylsulfanilamide
F. Maleylsulfathiazole
G. Malonylsulfathiazole
H. Succinylsulfamethyldiazine
I. Maleylsulfanilamide

Each compound is present in one millimolar concentration.

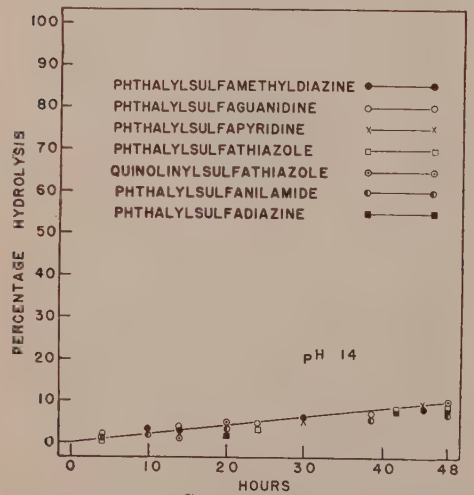


CHART III.

Graphic representation of the rates of hydrolysis of N^4 aromatic dibasic acid substituted sulfonamides in 1.0 N-NaOH at 38°C. Each compound is present in one millimolar concentration.

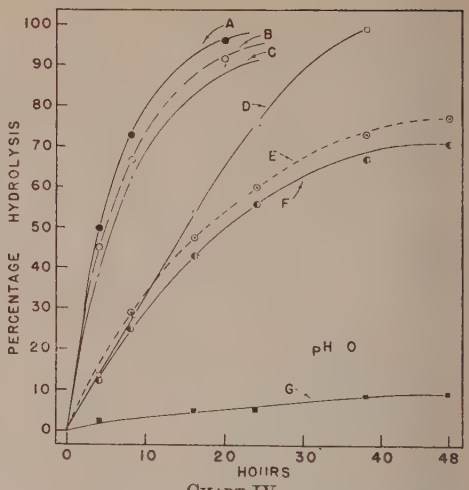


CHART IV.

Graphic representation of the rates of hydrolysis of N^4 aromatic dibasic acid substituted sulfonamides in 1.0 N-HCl at 38°C.

A. Phthalylsulfamethyldiazine
B. Phthalylsulfaguanidine
C. Phthalylsulfapyridine
D. Phthalylsulfathiazole
E. Quinolinylsulfathiazole
F. Phthalylsulfanilamide
G. Phthalylsulfadiazine

Each compound is present in one millimolar concentration.

of hydrolysis in acid are relatively rapid while the compounds are quite stable in alkaline solutions (Table I-C).

Considering succinylsulfathiazole, a representative of the saturated substituents, and phthalylsulfathiazole, a representative of the aromatic substituents, Chart V, it is evident that in the region of pH 7, the hydrolysis rate for each is slowest. (See also charts 7 and 8, pp. 365-6, Poth and Ross.[2])

Correlation of Hydrolysis Rate to Bacteriostatic Activity. If the antibacterial activity of these acylated sulfonamides is due to the free sulfonamide resulting from hydrolysis or deacylation, then the bacteriostatic activity of these compounds should be directly related to the tendency for deacylation to occur, Table I. This relationship should be even more evident in an homologous series of compounds such as is indicated in Table II. It will be noted, however, that such is not always true. The hydrolysis rates of succinylsulfathiazole and malonylsulfathiazole are essentially the same,

TABLE I.

Comparison of Rates of Hydrolysis, Bacteriostatic Activity, Concentration of Drug in Blood, and Toxicity of Various Conjugated Sulfonamides Following Oral Administration to the Dog.

The compounds are listed in 3 groups: *Group A*, the acylating radical is a saturated, dibasic organic acid; *Group B*, the acylating radical is an unsaturated, aliphatic, dibasic, organic acid; and *Group C*, the acylating radical is an aromatic, dibasic, organic acid. The rates of hydrolysis are determined in 1.0 N-HCl and 1.0 N-NaOH at 37°C. The bacteriostatic activity is the effect observed on the coliform organisms in the bowel of the dog following the oral administration of 0.167 g of drug per kilo of body weight every 4 hours. The toxicity and concentration of the drug in the blood are recorded for the same dosage level.

Conjugated sulfonamide	Relative rate of hydrolysis in normal		Relative bacteriostatic activity	Concentration of sulfonamide in mg per 100 cc of blood		Relative toxicity
	HCl	NaOH		Free	Conjugated	
Group A						
Succinylsulfaguanidine	++±	+++±	++±	—	—	0
Succinylsulfapyridine	++±	+++	+++	—	33.0	+++
Succinylsulfathiazole	++	+++	++++	3.5	5.0	0
Succinylsulfanilamide	++±	++±	++++	2.5	1.5	0
Succinylsulfamethyldiazine	+	++	++	—	—	0
Oxalylsulfathiazole	+++	+++±	+	—	—	+++
Malonylsulfathiazole	++	++±	±0	—	—	+++
Group B						
Maleylsulfathiazole	++++	++±	++++	10.95*	—	0
Maleylsulfanilamide	++++	+	++++	2.0	2.0	++++
Group C						
Phthalylsulfaguanidine	+++±	±	—	—	—	—
Phthalylsulfapyridine	+++±	±	—	—	—	—
Phthalylsulfamethyldiazine	+++±	±	0	4.3	5.0	0
Phthalylsulfathiazole	+++	±	+++++	3.0	3.0	0
Phthalylsulfanilamide	++	±	+	1.5	1.0	0
Phthalylsulfadiazine	±	±	0	4.3	1.0	0
Quinolinylsulfathiazole	++	±	++++	—	—	0

* Maleylsulfathiazole is so susceptible to acid hydrolysis that it is completely split in the course of the analytical procedure, which makes it impossible to determine the distribution of free and conjugated compounds in the blood.

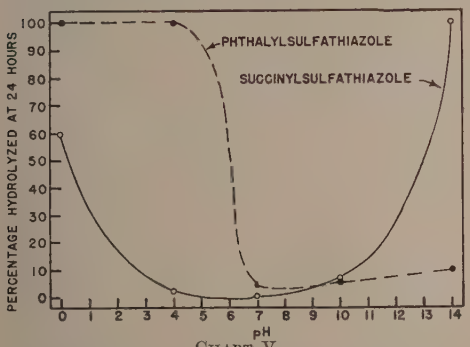


CHART V.

Comparison of the hydrolysis rates of succinylsulfathiazole and phthalylsulfathiazole at different hydrogen ion concentrations varying between that of 1.0 N-HCl and 1.0 N-NaOH at 38°C. The drugs are present in a concentration of 0.05 millimolar.

but succinylsulfathiazole is strongly bacteriostatic against the coliform organisms in the bowel of the dog while malonylsulfathiazole has only slight activity.

Likewise, the rate of hydrolysis of oxalylsulfathiazole in both acid and base greatly exceeds that of succinylsulfathiazole, and again the bacteriostatic action of the former is but a fraction of the latter compound.

The rate of hydrolysis in acid solution frequently parallels the bacteriostatic activity, but the rate of hydrolysis in basic solution gives no indication of the bactericidal action even in an homologous series of compounds. This latter fact is particularly emphasized by a comparison of quinolinylsulfathiazole, phthalylsulfathiazole, succinylsulfathiazole, and malonylsulfathiazole, Table II.

In the final analysis, of course, the rates of deacylation at the pH of the medium in which the drugs must act in the host is really the important consideration. In this connection the succinyl and phthalyl derivatives were incubated at 38°C with a suspension of human feces adjusted to pH 7, Table III. In this

TABLE II.

Comparison of Hydrolysis Rates and Bacteriostatic Activity of an Homologous Series of Acylated Sulfathiazole Derivatives.

These data are compiled from the same sources as those of Table I and were obtained under the same experimental conditions.

Acylated sulfathiazoles	Relative hydrolysis rates in normal HCl	Relative hydrolysis rates in normal NaOH	Relative bacteriostatic activity
Succinylsulfathiazole	++	+++	++++
Oxalylsulfathiazole	+++	+++±	+
Malonylsulfathiazole	++	++±	+
Maleylsulfathiazole	++++	++±	++++
Phthalylsulfathiazole	+++	±	++++
Quinolinylsulfathiazole	++	±	++++

TABLE III.

Rate of Hydrolysis of Acylated Sulfonamides in a Suspension of Human Feces.

0.2 millimoles of the respective compounds are mixed with 100 cc of a suspension containing 5.0 g of wet feces. The mixture is adjusted to pH 7 using a phosphate buffer and is incubated at 38°C. The initial coliform count is 110,000 per cc, which increased to approximately 10,000,000 per cc within the first 24 hours in each instance.

Acylated sulfonamide	Hours	Conc. of free sulfonamide in mg per 100 cc: 0	24	48	72	Initial conc. of conjugated drug in mg per 100 cc
Succinylsulfathiazole		1.3	5.6	6.9	11.9	71.0
Succinylsulfanilamide		1.4	4.3	5.7	10.0	54.6
Phthalylsulfathiazole		14.5*	15.8	15.6	13.1	80.6
Phthalylsulfanilamide		7.4*	6.7	7.4	6.9	64.0
Phthalylsulfadiazine		14.4*	12.5	15.0	15.0	79.8
Phthalylsulfamethyldiazine		16.8*	14.7	16.9	15.3	81.4
Phthalylsulfathiazole in buffer only		12.8*	13.5	14.0	13.5	80.6

* The relatively high values of free sulfonamide in these determinations are inherent in the analytical procedure, due to the rapidity with which the phthalyl derivatives are hydrolyzed in acid solution. Note that these values do not increase with incubation of the suspensions.

heterogeneous mixture of feces, containing many bacteria and various enzymes and ferments, succinylsulfanilamide and succinylsulfathiazole are appreciably hydrolyzed while the phthalyl derivatives show no tendency to be split. The finding that the phthalyl derivatives are chemically more stable is not to be expected from previous observations which show phthalylsulfathiazole to be more readily deacylated to maintain a higher concentration of free sulfonamide in the bowel than in the case of succinylsulfathiazole.

Discussion. An examination of the hydrolysis rates at which this series of conjugated sulfonamides undergo deacylation fails to indicate always the magnitude of the local, antibacterial activity of any single, individual, compound. These same facts do, however, again add some evidence to suggest that the entire antibacterial activity of these conjugated sulfonamides is not due solely to the action of free sulfonamides produced by the hydrolysis of the acylated derivative. From other observations,[12] we have postulated that the local bacteriostatic action of this series of compounds may arise from several possible sources: (1) the activity may be derived from the free sulfonamide liberated by hydrolysis;[1,14] (2) a portion of the activity may result from the physical and chemical properties of the conjugated compound which determine the local concentration of the unaltered drug adsorbed onto or absorbed into the individual susceptible microörganism, where; (3) the conjugated compound is acted upon by ferments within the cell to yield highly reactive split-products in a nascent or excited state, which products would in their turn enter

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into competitive reactions to prevent the formation of essential metabolites from specific substrates; and, (4) the activity may be influenced by the formation of split products not containing the sulfonamide radical such as succinate from succinyl derivatives. Since the antibacterial activity of members of an homologous series of these compounds does not parallel the ease with which sulfathiazole, for example, is formed by simple hydrolysis of the succinyl, phthalyl, quinolinyl, malonyl, oxalyl, and maleyl derivatives of sulfathiazole, it seems highly probable that the biologic activity is not explained entirely if it be assumed that such activity is due entirely to the sulfathiazole present.

Similarly, the high toxicity of maleylsulfanilamide as compared to the relatively low toxicity of maleylsulfathiazole cannot be explained upon the assumption that it is due to the split products, because sulfanilamide does not possess any such high degree of toxicity when compared to sulfathiazole.

Conclusion. Much of the data presented here cannot be correlated to show a relationship between antibacterial activity and rate of deacylation of the conjugated compounds, and it seems clear that the antibacterial activity of these compounds is not due solely to the liberation of the free sulfonamides. A considerable part of the antibacterial activity of these substances must, therefore, reside in the conjugated molecule. The node of action is complex and cannot be defined at this time.

The toxicity of the various compounds, likewise, does not necessarily parallel the chemical instability of the several conjugated sulfonamides.

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Effect of Steroid Substances on Synthesis of Acetylcholine.*

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Previous investigations have shown that male and female sex hormones may exert a vasomotor effect on the small vessels;[1-17] hormones of the adrenal cortex may change the size of the vessels due to changes induced in water and ion metabolism of the body;[18-20] estrogenic substances may increase the amount

* This study was aided by a grant from the Josiah Macy, Jr., Foundation.

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of acetylcholine in certain tissues such as uterus[6,21,22] and nasal mucosa;[23,24] estrogenic hormones may have a cholinergic effect;[25,26] and some of the steroid hormones modify the activity of choline esterase,[27] and the sensitivity of effector cells to acetylcholine.[27] In the following investigation it was ascertained whether or not steroid substances modify the synthesis of acetylcholine.

Method. The synthesis of acetylcholine was studied by the method of Quastel, Tennenbaum, and Wheatley[28] with minor modifications.[29] Varying amounts of the steroid substances were added to mixtures containing 100 mg minced fresh frog brain, 3 mg physostigmine salicylate, 4.8 mg glucose, and 3 cc Ringer's solution. The pH of the mixtures was adjusted to 7.4. Identical mixtures without the steroid substances served as controls. When oil solutions of the steroid substances were used the controls contained an equal amount of the solvent. The mixtures were shaken and incubated aerobically for 4 hours at 37°C. After incubation the amounts of free and total acetylcholine synthesized were assayed biologically on the sensitized rectus abdominis muscle of the frog. The amount of acetylcholine synthesized was calculated by subtracting from the acetylcholine content of the incubated mixtures the acetylcholine content of identical non-incubated mixtures. By adding the steroid substances in varying concentrations to incubated control mixtures after incubation it was ascertained whether or not the substances modified the sensitivity of the rectus abdominis muscle to the acetylcholine content of the mixtures during the 2 minutes of immersion for biological assay. If so, the potentiation was taken in account by the calculation.

Results. The amounts of acetylcholine synthesized in the presence of 23 steroid substances are given in Table I. Within the range of concentrations used only the estrogenic substances and pregnenolone increased the synthesis of acetylcholine. These results offer some explanation of the mechanism of the synergistic action of physostigmine with estrogenic substances in inducing menstruation.[30,31] The failure of Emmens, MacIntosh, and Richter[32] to demonstrate changes in the synthesis of acetylcholine in the presence of estradiol was probably due to the use of relatively high concentrations of estradiol.

The other substances either did not modify the synthesis of acetylcholine (cholesterol), or did not modify it in low concentrations and depressed the synthesis in higher (bile salts, vitamin D), or depressed the synthesis in low and increasing concentrations. Therefore, should these substances have any acetylcholine-like effect it is not because of the increase of synthesis of an acetylcholine-like agent in the body.

Summary and Conclusions. 1. The effect of steroid substances on the synthesis of acetylcholine was investigated. 2. Cholesterol did not modify the synthesis of acetylcholine. 3. Bile salts and vitamin D did not modify the synthesis in low concentrations and depressed it in higher. 4. Hormones of the adrenal cortex, male sex hormones, and corpus luteum hormones, except pregnenolone, depressed the synthesis in low and increasing concentrations. 5. Estrogenic hormones and

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TABLE I.
Effect of Steroid Substances on the Synthesis of Acetylcholine.

Substance	Solvent	No. of series of exper.	Amt of acetylcholine synthesized in % of control*: Amts of substances added to 100 mg frog brain (in mg) 3	0.3	0.03	0.003	Free acetylcholine	3	0.3	0.03	0.003	Total acetylcholine
Cholesterol		10	108	107	102	105		98	99	97	107	
Bile salts†		8	25	83	97	96		31	85	90	94	
Vitamin D‡		8		30	75	97			32	77	95	
Desoxycorticosterone acetate (Ciba)		6	55	67	78	85		42	61	77	76	
" " (Schering)		6	50	60	69	83		55	59	68	82	
4,5-dihydro-17-hydroxy-11-dehydro-corticosterone (Upjohn)		3	39	57	81	83		38	65	71	80	
Androstenedione (Schering)		8	27	40	64	90		29	35	59	88	
Dehydroandrosterone acetate (Schering)		8	60	79	89	95		60	71	85	91	
Testosterone (Schering)		8	55	77	81	89		47	71	86	90	
" propionate (Ciba)		8	53	66	75	85		57	74	81	86	
Methyltestosterone (Ciba)		8	28	48	72	96		35	50	70	94	
Δ^5-pregnenolone (Schering)		8	124	127	130	120		122	125	127	119	
Progesterone (Ciba)		10	62	68	84	91		61	71	82	87	
Pregnandiol (Ayerst, McKenna, & Harrison)		8	75	83	87	91		73	80	85	91	
Sodium pregnandiol glucoronidate (same)		5	53	64	75	80		56	60	73	82	
Allo-pregnanedione-11,20-diol-3,21 (Upjohn)		3	31	37	76	85		30	37	65	80	
Etiochilonol-3α-11-one acetate (Ayerst, McKenna, & Harrison)		5	65	80	100	110		73	86	115	121	
Estrone (Upjohn)		3	160	155	150	140		157	153	149	137	
Estradiol (Ciba)		8	103	110	125	130		102	111	127	129	

			Amts of substances added to 100 mg frog brain (in cc) 0.5	0.1	0.05	0.01	0.001	0.5	0.1	0.05	0.01	0.001
Doca (Roche-Organon) (1)§	Sesame oil	12	40	45	51	57		34	50	55	58	
Oreton (Schering) (2)§	" "	12	77	77	75	74		70	72	74	76	
Progestin (Roche-Organon) (3)§	Peanut "	12	63	65	71	77		63	69	74	76	
Estrone suspension (Abbott) (4)§	Acacia	12	100	122		138	137	110	130		139	141

* The S.E. of the mean for each value was less than $\pm 4\%$. The amounts of acetylcholine synthesized in μg per 100 mg frog brain, followed by the S.E. of the mean were: Free acetylcholine, 0.70 ± 0.023; total acetylcholine, 1.50 ± 0.044.

† Sodium glycocholate and sodium taurocholate from fresh ox gall (Fairchild Bros. & Foster).

‡ Each mg contained 400 I.U. vitamin D. The synthesis of acetylcholine was not modified in the presence of vitamin D in concentrations from 0.003 to 0.00001 mg.

§ One cc of the original solution contained (1) 5 mg desoxycorticosterone acetate; (2) 5 mg testosterone propionate; (3) 1 mg progesterone; (4) 2 mg estrone.

pregnenolone increased the synthesis of acetylcholine.

The authors wish to express their gratitude to Abbott Laboratories, Ayerst, McKenna & Harrison (U.S.), Ltd., Ciba Pharmaceutical Products, Inc., Roche-Organon, Inc., Schering Corporation, and Upjohn Company for the generous supply of hormones.

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Dietary Cirrhosis without Ceroid in Rats.

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Lillie, Daft, and Sebrell[1] among others have reported the production of hepatic cirrhosis in rats given a purified diet low in protein and low in choline. In their report and in the reports of other investigators[2,3] it was noted that the cirrhosis was always accompanied by the deposition of considerable amounts of a peculiar insoluble pigment in the fibrous trabeculations of the cirrhotic livers. The name "ceroid" has been given to this pigment.[4] Endicott and Lillie[5] have described in detail its staining properties and have noted that it is insoluble in all common solvents. Ceroid has not been reported in human liver cirrhosis nor in the experimental cirrhosis produced in the dog, the guinea pig, the pig, or the rabbit.

The question of whether or not ceroid is an essential part of the cirrhosis in rats has been the subject of much speculation. Ceroid was found to resemble the lipoid residues in cod liver oil pneumonia and similar substances were produced by chromate oxidation of cod liver oil and linseed oil but not by oxidation of various hydrogenated oils.[6] This suggested that dietary cirrhosis without ceroid might be produced by altering the dietary fat. Together with the work of Hass,[7] it suggested also the possibility that fatty acids found in both vegetable and animal oils and possessing more than one double bond might be involved in ceroid production. We have now achieved the production of cirrhosis without ceroid. Our results seem to indicate, however, that the most important dietary precursor of ceroid is not an ingredient of Wesson oil and that it is distinct from linoleic and linolenic as well as oleic, palmitic and stearic acids. It appears probable that the substance in question is present in cod liver oil but we have not as yet identified it further.

Weanling albino rats were given one of several purified diets containing leached and alcohol-extracted casein 4%, cystine 0.5%, and salt mixture No. 550[8] 4%. Diet No. 895 contained no fat or fatty acid, diet No. 954 contained 5% of Wesson oil, diet No. 953 contained 1% of a mixture of oleic, linoleic, linolenic, and saturated acids,* and

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* Armour and Company's "Neo-Fat No. 23." The composition given by the Company is oleic acid 30%, linoleic acid 56%, linolenic acid 10%, and saturated acids 4%.

diets No. 950, No. 951, and No. 952 contained 10% of palmitic, stearic and oleic acids respectively. Sucrose made up the remainder of the diets. Each rat received a daily supplement of 100 μg of thiamine hydrochloride, 50 of riboflavin, 20 of pyridoxine hydrochloride, 50 of calcium pantothenate and 1 mg of niacin. Each rat also received a weekly supplement of 3 mg of α-tocopherol in ethyl laurate and a bi-weekly supplement of 500 units of vitamin D in propylene glycol and 100 μg of carotene (S.M.A.) in ethyl laurate. A few of the rats on the fat-free diet received 20% alcohol *ad libitum* as the sole source of fluid; all of the other rats received water *ad libitum*. The animals were kept on these diets until their deaths which ranged from 10 days to 194 days from the beginning of the experiment (average of 56 animals on experiment, 66 days).

Histological examination of the liver, spleen, kidney, and adrenals of these 56 animals failed to reveal any trace of ceroid. Two of 5 of the animals receiving Wesson oil (deaths at 63 and 118 days), 1 of 5 receiving the mixture of oleic, linoleic, linolenic, and saturated acids (death at 110 days), 1 of 23 receiving the fat-free diet with water to drink (death at 111 days), and 2 of 8 receiving the fat-free diet and alcohol (deaths at 48 and 93 days) developed cirrhosis of the liver. None of the 15 rats receiving oleic, palmitic, or stearic acids (5 on each diet) developed cirrhosis but this may have been due to the short period of survival of these animals. Only 2 lived as long as 50 days on the experiment and the last survivor died at 70 days.

The cirrhotic livers showed fibrous trabeculation and distortion of lobular architecture. The distribution of the trabeculation was similar to that described by Lillie and coworkers.[4] The fibrous trabeculæ appeared to connect centrolobular areas, occasionally coursing through or near portal structures. Most liver cells contained large globules of fat.

It appears likely that the presence of cod liver oil in the ceroid-producing diets such as diet No. 545 employed in previous experiments[9,10,11] constitutes the important difference between these diets and the diets used in the present experiments, which did not cause the deposition of ceroid. Diet No. 954, containing 5% Wesson oil is very similar to diet No. 545 containing 2% cod liver oil and 3% Wesson oil. Diet No. 545 contains cornstarch instead of sucrose and Osborne and Mendel salt mixture instead of modified O. and M. salt mixture No. 550, but we have from time to time used many cirrhosis-producing diets containing sucrose and salt mixture No. 550, and have found that ceroid invariably accompanies the cirrhosis (unpublished experiments). In this connection the following statement by György[11] should be noted: "A survey of the nutritional conditions under which ceroid in experimental cirrhosis in rats was noted, reveals the presence of cod liver oil and the absence of large amounts of yeast or of other crude sources of the vitamin B-complex in the diet."

Although these results are clearly preliminary in nature, several points of interest are indicated. Perhaps first in importance is the fact that it is possible to produce dietary cirrhosis in rats without accompanying ceroid deposition. A second point of interest is the indicated probability that ceroid is related to some substance or substances present in cod liver oil. It is of some further interest that cirrhosis may be produced in rats on diets completely devoid of fat or fatty acids.

It is not clear as yet whether or not the formation and deposition of ceroid affects the incidence and severity of cirrhosis.

Conclusions. 1. Hepatic cirrhosis without ceroid has been produced in rats by feeding certain purified diets. 2. The type of fat given to rats in low-choline, low-protein diets appears to have an important influence on the deposition of ceroid. It seems probable that some substance or substances which are present in cod liver oil causes the appearance of this pigment. We have not observed ceroid in rats receiving palmitric, stearic, oleic, linoleic, or linolenic acids. 3. Cirrhosis may be produced on a completely fat-free diet.

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Mammary Development in the Thyroprived Bovine by Stilbestrol and Thyroprotein Administration.

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The role of the thyroid in mammary gland development and lactation is still controversial. In rats Nelson[1] and Nelson and Tobin[2] report no observable effect while Folley[3] and Karnofsky[4] observed a marked diminution in lactation after thyroidectomy. Leonard and Reece[5] reported thyroidectomy to enhance mammary growth from estrogen therapy in female rats whereas Smithcors and Leonard[6] reported the opposite for male mice and Mixner and Turner[7] report improved mammary growth in thyroprived rats from estrogen and progesterone administration.

Reports of mammary development in thyroidectomized cows and goats are all based upon observations on lactation following gestation. While most workers report a varying depressing effect on lactation following thyroidectomy all observed mammary development as based upon subsequent lactation. In all of these reports, however, the presence of thyroxine from fetal secretions was not ruled out. Spielman, Petersen, and Fitch[8] observed that myxedematous symptoms of thyroidectomized cows began to disappear about midterm due presumably to secretions from the developing fetus or fetal membranes. Although the mammary glands were not as well developed nor lactation as great in the thyroprives as in the normals because of the unknown part fetal secretions might play, observations in these experiments can be interpreted as only indicating the thyroid is concerned with full mammary development.

Since it is established that diethylstilbestrol administration to the non-pregnant cow mediates both mammary growth and lactation somewhat comparable to that following a normal gestation, the use of this drug on thyroprived cows suggests itself as a logical approach to a solution of the problem insofar as this species is concerned. Accordingly 2 thyroidectomized cows, A26 and 823, with marked clinical myxedema, were subjected to diethylstilbestrol administration first alone and then together with thyroid replacement therapy.

A26 was a unipara thyroidectomized when 13 months of age, that conceived by artificial insemination at 21 months of age when markedly myxedematous and carried a normal male fetus to term. About midpregnancy myxedematous symptoms began to disappear and the blood cholesterol became normal. On the basis of comparisons with normal sibs her mammary glands appeared to be about half normal in size at term and initial milk production about one-half that expected. Lactation rapidly declined with cessation at 190 days and an apparently completely involuted mammary gland.

Over a period of 2 years A26 was subjected to a series of 10 experiments. Following experiments in which mammary development occurred or recovery from the myxedematous symptoms sufficient rest periods were allowed for complete involution of the mammary gland and return to the marked myxedematous state. Numbered chronologically, in experiments 1, 3, 6, and 7, she was treated on alternate days with 30 mg diethylstilbestrol. The length of treatment was 31 and 21 days respectively for the first and second 2 of these experiments.

In experiment 2 four ounces of fresh raw

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2 Nelson, W. D., and Tobin, C. E., *Anat. Rec. Suppl.*, 1937, **67**, 111.

3 Folley, S. J., *J. Physiol.*, 1938, **93**, 401.

4 Karnofsky, D., *Endocrinology*, 1942, **30**, 234.

5 Leonard, S. L., and Reece, R. P., *Endocrinology*, 1941, **28**, 65.

6 Smithcors, J. F., and Leonard, S. L., *Endocrinology*, 1942, **31**, 554.

7 Mixner, J. P., and Turner, C. W., *Endocrinology*, 1942, **31**, 345.

8 Spielman, A. A., Petersen, W. E., and Fitch, J. B., *J. Dairy Science*, 1944, **27**, 441. Also unpublished data.

thyroid was given daily and 30 mg diethylstilbestrol on alternate days for a period of 29 days. In experiment 3 covering 21 days, 18 g thyroprotein was given daily with 30 mg diethylstilbestrol on alternate days.

In experiments 4 and 9 10 g of thyroprotein were given daily without diethylstilbestrol.

Experiments 5 and 10 were continuations respectively of 4 and 9 with 30 mg of stilbestrol administered on alternate days for 16 days in the former and 29 days in the latter. Thyroprotein administration continued for several months after withdrawal of the diethylstilbestrol treatment.

The effect of diethylstilbestrol alone in the myxedematous state and together with thyroprotein after the myxedematous symptoms had been relieved were also studied in 823, a Holstein female that had been thyroidectomized at 17 months of age. Marked myxedematous symptoms developed in 145 days, post-operatively, before 20 mg diethylstilbestrol were administered on alternate days for 29 days. Next 10 g thyroprotein were administered orally per day for 30 days followed by 20 mg stilbestrol on alternate days for 19 days. The smaller dosage of diethylstilbestrol is occasioned by 823's smaller size.

In the 5 experiments in which diethylstilbestrol was administered to thyroidectomized myxedemic cows there was no evidence of any effect upon mammary development that could be detected by careful palpation. The doses of diethylstilbestrol used were larger and the period of time over which it was administered longer than has been shown necessary to obtain marked mammary development and lactation in normal nonlactating cows.

In the 3 periods when either raw thyroid or thyroprotein were administered there was no discernible mammary development. There were, however, other marked physiological changes. Gradually activity and excitability increased to that of the normal animal. Heart and respiration rates also attained normalcy by the end of 30 days of thyroid therapy. Although appetite increased there was a loss in weight thought to be due largely to the disappearance of edema.

In the 3 experiments in which fresh thyroid or thyroprotein were administered to pronounced myxedematous subjects no mammary development was observable until there was a marked recovery from clinical myxedema and then the development was slow.

In the 3 experiments in which 30 mg of diethylstilbestrol was administered together with thyroprotein, after recovery from clinical myxedema had been effected, mammary development was rapid being distinctly noticeable in 5 to 7 days. Milk secretion was first observed on the 13th day in experiments 5 and 10 and on the 18th day for 823 following the beginning of diethylstilbestrol administration. At first the secretion amounted to but a few ml per day but increased rapidly. The milk production for each of the 3 experiments is given in Table I. The second lactation of A26 and the one for 823 is still in progress. The first lactation of A26 was deliberately terminated in order to prepare her for subsequent experiments.

Although the lactation level is not nearly that which would be expected following normal parturition of normal animals, it is substantial. Although no evidence of mammary development could be detected from administration of diethylstilbestrol in the myxedematous state and substantial development and lactation followed administration of this drug together with thyroprotein subsequent to 30 days of previous thyroid therapy it can not be concluded that thyroxine and stilbestrol act synergistically to promote mammary development. Complete failure of mammary development when these 2 substances were administered simultaneously to myxedematous animals until there was marked clinical improvement, must be considered as *a priori* evidence against such a synergistic relationship.

Since the mammary response to diethylstilbestrol treatment began when relief from myxedema became apparent, it appears that some phase of the myxedema syndrome is responsible for the failure of mammary growth. The lowered metabolism and arrest of growth which follows thyroidectomy could well account for the failure of mammary response to stilbestrol as mammary development is a growth phenomenon and has been shown by Astwood, Geschickter, and Rausch[9] to fail in

[9] Astwood, E. B., Geschickter, C. F., and Rausch, E. O., *Am. J. Anat.*, 1937, **61**, 373.

TABLE I.

Record of 3 Lactations Induced by the Simultaneous Administration of Diethylstilbestrol and Thyroprotein to Thyroidectomized Cows Following 30 Days Previous Treatment with Thyroprotein.

A26 Experiment 5		A26 Experiment 10		823	
1943	Milk (lbs.)	1944	Milk (lbs.)	1944	Milk (lbs)
Feb. 19 da.	36.4	May 13 da.	51.1	June	9.1
Mar.	255.9	June	129.2	July	163.4
April	304.3	July	197.8	Sept.	222.8
May	286.0	Aug.	370.0		
June	189.0	Sept.	432.1		
July	206.0				
Aug. 3 da.	16.1				
	1294.0				

inanition in the rat.

Summary. In the thyroprived cow with pronounced myxedema no mammary development was obtained by diethylstilbestrol or thyroprotein administration. Negative results were also obtained with the simultaneous administration of these two substances as long as myxedema persisted. Substantial mammary development and lactation resulted from diethylstilbestrol administration following recovery from myxedematous symptoms by thyroprotein therapy. It is suggested that the failure of mammary development in the myxedematous animal is due to some phase of the myxedema and that the response obtained following thyroid therapy is not due to a synergistic action of thyroxine and diethylstilbestrol.

The diethylstilbestrol was furnished by Merck and Company through the courtesy of Dr. D. F. Green. The thyroprotein was furnished by the Cerophyll Company through the courtesy of Dr. R. Graham and the L. Perrigo Company through the courtesy of Dr. L. C. Curlin.

14802

Urethane Administration and Potassium Content of Bronchial Secretions in the Cat.

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During the course of studies upon the chemical composition of respiratory tract fluid (R.T.F.), part of which has been published,[1,2] it has been found that the potassium content of these bronchial secretions is higher in cats under urethane anesthesia than in decerebrate cats. This observation is of coincidental interest because (a) potassium has been found generally[3,4,5,6] though not always[7] related in some obscure manner to sympathomimetic activity; (b) urethane has been reported

1 Perry, W. F., and Boyd, E. M., *J. Pharm. and Exp. Therap.*, 1941, **73**, 65.

2 Boyd, E. M., Jackson, S., MacLachlan, M., Palmer, B., Stevens, M., and Whittaker, J., *J. Biol. Chem.*, 1944, **153**, 435.

3 Camp, W. J. R., and Higgins, J. A., *J. Pharm. and Exp. Therap.*, 1936, **57**, 376.

4 Brewer, G., Larson, P. S., and Schoeder, A. R., *Am. J. Physiol.*, 1939, **126**, 708.

5 Larson, P. S., and Brewer, G., *J. Pharm. and Exp. Therap.*, 1937, **61**, 213.

6 Brewer, G., and Larson, P. S., *J. Pharm. and Exp. Therap.*, 1938, **63**, 272.

7 Seager, L. D., *J. Pharm. and Exp. Therap.*, 1939, **66**, 202.

to relax bronchial muscles and to be useful in the therapy of bronchial asthma,[8] and (c) potassium salts have been found to be effective in the treatment of bronchial asthma by some (*e.g.* [9]) but not by others (*e.g.* [10]). These various observations would suggest the possibility that urethane relaxes the bronchial muscles of Reisseissen by setting free in the respiratory tract potassium ion which, by virtue of its sympathomimetic activity, relaxes these muscles.

The experiments were performed upon 32 healthy, adult cats, 17 of which were decerebrated and 15 urethanized by a parenteral injection of 1 g of ethyl carbamate per kilo body weight. The animals were arranged for collection of R.T.F. by the method of Perry and Boyd,[1] as modified by Boyd, Jackson, and Ronan.[11] The volume output of R.T.F. was identical in the 2 groups of animals and averaged about 2 ml per kilo body weight per 24 hours. The R.T.F. excreted during the first 3 hours, from the 4th to the 12th and from the 13th to the 26th hour, was separately analyzed for potassium by the method of Hoffman,[12] the results being expressed as mg of potassium per 100 ml of R.T.F.

TABLE I.

Mean Potassium Content of R.T.F., Expressed as mg per 100 ml, in Urethanized and Decerebrate Cats.

Hours of collection of R.T.F.	Urethanized	Decerebrate
1 to 3	28	5.6
4 to 12	18	9.2
13 to 26	36	8.1
Mean	27	7.6

[8] Farmer, L., *J. Lab. Clin. Med.*, 1939, **24**, 453.

[9] Bloom, B., *J. Am. Med. Assn.*, 1938, **111**, 2281.

[10] Miller, H., and Piness, G., *J. Am. Med. Assn.*, 1940, **114**, 1742.

[11] Boyd, E. M., Jackson, S., and Ronan, A., *Am. J. Physiol.*, 1943, **138**, 565.

[12] Hoffman, W. S., *J. Biol. Chem.*, 1937, **120**, 57.

The results have been averaged and the mean values are given in Table I. It is obvious that the output of potassium is considerably greater, on the average 3 to 4 times as great, in the R.T.F. of the urethanized than of the decerebrate cats.

There remained the possibility that urethane, by increasing the concentration of serum potassium, caused an increased output of potassium in the R.T.F. Anesthesia has in general been found associated with a fall in the level of serum potassium, although in species with large amounts of potassium in the red blood cells, hemolysis may complicate the results and give false high values for serum potassium during anesthesia.[13] To investigate this possibility, we analyzed the serum potassium of 8 cats under urethane anesthesia, taking blood by cardiac puncture at 3-hour intervals. The initial pre-anesthetic concentration of serum potassium averaged 21.5 mg per 100 ml of serum; at 3 hours, the average value was 19.0 mg, at 6 hours it was 17.8 mg, and at 9 hours it averaged 19.9 mg per 100 ml. These results are in conformity with those previously published in demonstrating that anesthesia produces a slight fall in the concentration of serum potassium and obviously could not be used in any way to explain the changes found in the potassium content of R.T.F.

Summary. The potassium content of respiratory tract fluid was found to average 7.6 mg per 100 ml in decerebrate cats, and 27 mg per 100 ml in urethanized cats. The results could not be related to serum potassium, the level of which declined slightly during urethane anesthesia; it is suggested that they may have some relation to the reputed value of urethane in bronchial asthma and the sympathomimetic action of potassium.

[13] Robbins, B. H., and Pratt, H. A., *J. Pharm. and Exp. Therap.*, 1936, **56**, 205.

14803

Cross-Reacting Typhus Antibodies in Rocky Mountain Spotted Fever.

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It is generally accepted that the rickettsial diseases of man may be separated into 4 distinct groups according to their immunological relationship. (Trench fever is not included since its etiology has not been definitely established.) While the members of the typhus group, the spotted fever group, the scrub typhus group, and the "Q" fever group confer an immunity to the diseases of their respective group, most observers consider that there is no cross immunity between the groups. However, an antigenic relationship between members of the typhus and spotted fever groups of diseases is suggested since both groups are characterized by the presence of an OX-19 agglutination. This hypothesis was investigated by Castaneda and Silva[1] who observed that when typhus-immune guinea pigs were challenged with a virulent strain of Rocky Mountain spotted fever, a febrile reaction of variable duration occurred with only 4 of 17 animals dying while all of the 19 normal control guinea pigs developed fever and died. While no complete cross immunity was demonstrated, these experiments did indicate that typhus-immune guinea pigs developed a partial immunity to Rocky Mountain spotted fever. The same authors performed rickettsial agglutination tests, using a suspension of murine rickettsiæ obtained from infected rat lungs and convalescent human serum from cases of Rocky Mountain spotted fever. Two macroscopic agglutination tests showed complete agglutination at titers of 1/160 and 1/20 respectively while the third specimen showed partial agglutination at 1/20. Microscopic agglutination tests performed on 6 specimens of human convalescent Rocky Mountain spotted fever serum with murine rickettsiæ were recorded as showing some agglutination. The sera of infected Rocky Mountain spotted fever guinea pigs likewise were tested with murine rickettsial suspensions and it was observed that no agglutination developed from the 1st to the 6th day of disease while specimens obtained from the 6th to the 31st day showed more or less agglutination, but none was observed after 32 days. From the experimental data presented the authors concluded that there was a definite immunological relationship between Rocky Mountain spotted fever and typhus.

Parker[2] observed in cross immunity tests in guinea pigs that while animals that had recovered from murine typhus were fully susceptible when inoculated with a strain of Rocky Mountain spotted fever isolated from *Amblyoma americanum*, the converse was not true. Of 4 guinea pigs recovered from Rocky Mountain spotted fever and challenged with a strain of murine typhus, 2 guinea pigs remained afebrile and 2 had 3 and 6 days of fever respectively, without the scrotal swelling noted in the controls. In similar studies with epidemic typhus versus Rocky Mountain spotted fever, of 15 guinea pigs recovered from epidemic typhus and challenged with Rocky Mountain spotted fever 6 guinea pigs remained afebrile and 9 developed fever of from one to 6 days' duration. In the reverse test, when 12 Rocky Mountain spotted fever immune guinea pigs were challenged with an epidemic strain, 4 remained afebrile and 8 developed fever of from 2 to 5 days' duration. It is apparent from these observations that a partial immunity between typhus and Rocky Mountain spotted fever was present, at least with the strains investigated.

The following study was undertaken to obtain further serological data on the possible immunological relationship between these two diseases.

A series of 32 serum samples were examined

* Member of the U. S. A. Typhus Commission.

1 Castaneda, M. R., and Silva, R., *J. Immunol.*, 1941, **42**, 1.

2 Parker, R. R., *Public Health Rep.*, 1943, **58**, 721.

TABLE I.
Serological Tests on Specimens from Cases of Rocky Mountain Spotted Fever.

Case No.	Day of Disease	Weil-Felix*		Complement fixation†			Rickettsial Agg.*		Mouse neut. 50% endpoint*
		OX-19	OX-2	RMSF	Epidemic	Murine	Epidemic	Murine	Epidemic
1	7	1/160	0	0	0	0	1/40	1/160	‡
	14	1/640	0	1/160	0	0	1/640	1/640	6510
	15	1/640	0	1/160	0	0	1/80	1/2560	1444
	21	1/640	0	1/320	0	0	1/80	1/320	‡
2	12	0	0	0	0	0	0	0	0
	16	1/2560	1/160	1/40	0	0	‡		64
	106	1/80	1/40	1/80	0	0	0	1/80	23
3	16	0	0	1/20	0	0	0	0	2888
		Fatal-strain isolated							
4	6	0	0	0	0	0	0	1/80	—
	49	1/320	1/40	1/40	0	0	0	1/160	6
5	44	0	0	1/80	0	0	1/40	1/80	—
	217	1/40	0	1/80	0	0	0	0	8
6	30	1/320	1/320	1/80	0	0	‡		—
	33	1/320	1/160	1/160	0	0	0	1/80	—
	40	1/320	1/320	1/160	0	0	0	1/80	45
	75	1/160	1/160	1/160	0	0	0	1/80	13
	155	1/320	1/160	1/640	0	0	0	1/40	—
7	4	0	0	1/10	0	0	0	1/20	12
8	35	0	1/80	1/40	0	0	1/40	1/40	—
	146	0	1/20	1/80	0	0	0	1/20	45
9	57	1/160	0	1/640	0	0	0	1/20	204
	120	1/20	1/20	1/1280	0	0	0	1/80	132
10	130	1/40	1/80	1/80	0	0	0	1/80	128
11	5	0	0	0	0	0	0	0	—
	12	1/640	0	1/80	0	0	1/80	1/160	—
	64	1/160	1/20	1/320	0	0	0	1/40	—
	75	1/80	0	1/320	0	0	0	0	16
12	30	0	1/640	1/1280	0	0	0	1/40	—
	71	0	1/160	1/1280	0	0	0	0	—
13	44	0	1/160	1/640	0	0	1/80	0	—
	96	0	1/160	1/320	0	0	1/40	1/20	—
14	29	1/160	1/2560	1/80	0	0	0	1/40	—

* Final dilution.
† Initial dilution.
‡ Not sufficient serum.

from 14 cases of Rocky Mountain spotted fever. Complement fixation tests for Rocky Mountain spotted fever,[3] epidemic and murine typhus,[4] rickettsial agglutination for epidemic and murine typhus,[5] Weil-Felix tests and epidemic neutralization tests[6,7] were performed on each specimen of serum. The results in Table I were observed.

It is noted that a positive complement fixation test for Rocky Mountain spotted fever was obtained some time or other during the

[3] Plotz, H., and Wertman, K., *Science*, 1942, **95**, 445.

[4] Plotz, H., *Science*, 1943, **97**, 20.

[5] Plotz, H., and Snyder, M., Report to the Surgeon General (withheld from publication).

[6] Gildermeister, E., and Haagen, E., *Deut. Med. Wchnschr.*, 1940, **66**, 878.

[7] Henderson, R. G., and Topping, N. H., *Public Health Rep.*, 1944, 58 (withheld from publication).

course of the disease in the 14 cases studied. In 6 cases a rise in complement fixation titer was found when early and late specimens were compared. The complement fixation reactions with an epidemic and murine rickettsial antigen were all negative.

Macroscopic agglutination tests, performed with purified epidemic and murine rickettsial suspensions obtained from yolk sac cultures, were made on 30 specimens and agglutinins were demonstrable in 24. Five cases showed both epidemic and murine agglutinins, 8 showed murine agglutinins alone and one early case showed no agglutination whatsoever. In 5 cases there was a rise in titer when early and late specimens were compared. The titers varied from 1/20 to 1/2560. The reactions were clear cut and represent complete agglutination at the titer recorded.

Mouse neutralization tests were performed with a toxic substance derived from yolk sac cultures of epidemic typhus. Sixteen specimens were tested for the presence of neutralizing antibody and positive tests were demonstrated in 15. The 50% endpoint titers (Reed and Muench) varied from 6 to 6510. In 3 cases rather high titers were found, the 50% endpoint being 6510, 2888, and 1444.

The results of the Weil-Felix tests were in accord with those reported by Davis and Parker.[8] In the 14 cases of Rocky Mountain spotted fever studied, the sera of 5 agglutinated Proteus OX-19 at a higher dilution, 3 agglutinated Proteus OX-2 at a higher dilution, and one agglutinated equally well with both Proteus OX-19 and OX-2. Significant titers were not obtained in the remaining five cases. One was an early fatality, 3 were obtained late in convalescence, and one was a single specimen obtained early in the febrile period.

These results indicate that epidemic and murine rickettsial agglutinins and epidemic neutralizing antibodies may occur in some convalescent specimens of Rocky Mountain spotted fever.

The presence of typhus antibodies in cases of Rocky Mountain spotted fever raises the question as to whether these antibodies were due to a previous attack of typhus fever or immunization with typhus vaccine. Since the specimens examined came from military personnel where immunization records are kept, we have verified that none had received typhus vaccine nor was there a history of a previous infection of typhus fever.

[8] Davis, G. E., and Parker, R. R., *Public Health Rep.*, 1932, **47**, 2.

TABLE II.
Epidemic Typhus Neutralizing Antibody in Late Convalescent Specimens from Epidemic Typhus Fever.

Case No.	Day of disease	Neutralizing antibody titer 50% endpoint
1344	286	51
1345	117	102
1495	137	180
1894	318	81
1902	99	256
2724	286	323
3558	251	45
3732	245	722
4569	104	814
4956	235	814
5038	241	51
5043	276	2888
5585	229	323
5587	226	202
6243	218	40
7932	209	90

TABLE III.
Epidemic Typhus Neutralizing Antibody in Specimens from Rocky Mountain Spotted Fever.

Case No.	Day of disease	Neutralizing antibody titer 50% endpoint
1	14	6510
	15	1444
2	12	0
	16	64
	106	23
3	16	2888
4	49	6
5	217	8
6	40	45
	75	13
7	4	12
8	146	45
9	57	204
	120	132
10	130	128
11	75	16

In view of the fact that one could be tempted to employ the neutralization test to demonstrate the existence of a past infection, it was of interest to compare the 50% endpoint neutralizing antibody titers against epidemic typhus obtained in late convalescent specimens from proven cases of epidemic typhus fever with those found in specimens from cases of Rocky Mountain spotted fever. The results are given in Tables II and III.

If we accept the figure 40 as representing the lowest 50% endpoint neutralizing titer (case 6243—strain isolated) which occurred in late convalescence in our series of epidemic typhus fever cases, it is observed that 9 of the 16 specimens studied from cases of Rocky Mountain spotted fever gave higher titers. As already pointed out three of the Rocky Mountain spotted fever specimens gave titers of epidemic neutralizing antibody as high as or higher than were found in late convalescent specimens from cases of epidemic typhus fever. These results must be taken into consideration if the neutralization test is used for survey purposes.

Conclusion. Serological studies on convalescent specimens from cases of Rocky Mountain spotted fever show the presence of epidemic and murine agglutinins and epidemic neutralizing antibodies although there is no evidence of cross complement fixation. This evidence fits in with the observations of Castaneda and Silva and those of Parker, and indicates that there is a serologic relationship between epidemic typhus fever and Rocky Mountain spotted fever. The data presented in this paper must be considered in evaluating the results obtained with rickettsial agglutination or the neutralizing antibody when these tests are used as diagnostic procedures.

14804

pH of Nasal Mucosa of Some Laboratory Animals.

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The susceptibility of individuals to infections of the respiratory tract shows seasonal variations. This also appears to be true of some of the laboratory animals. A number of physiological factors are involved in the defense mechanism against bacterial and viral invasions, and in determining the severity of the infection. Of these factors, the pH of the nasal mucosa may play a role. There is disagreement as to what constitutes the normal pH of nasal mucosa. Using a glass electrode *in situ*, Fabricant[1] reported a surprisingly low average value of 6.2 for man with large fluctuations. Nungester and Atkinson[2] pointed out several errors in the method used by Fabricant. Their determinations yielded a value of pH = 7.0. Neither investigator recorded the temperature at which the readings were made. They also introduced a saline bridge without testing the effect of the additional boundary potentials. The effect of these boundary potentials has, however, been shown to be small.[3]

Hamsters were selected for the study of pH of nasal mucosa because the animals are susceptible to some viral diseases. Several readings were also made using rats and ferrets. This article deals with normal values. Special glass electrodes were constructed so that the entire glass membrane would be in contact with the nasal mucosa. The saturated calomel cell made direct contact with the animal through an asbestos-fiber wick.

Methods. The glass electrodes were made

[1] Fabricant, N. D., *Arch. Otolaryng.*, 1941, **34**, 150.

[2] Nungester, W. J., and Atkinson, A. K., *Arch. Otolaryng.*, 1944, **39**, 342.

[3] Voegtlin, C., Kahler, H., and Fitch, R. H., *Nat. Inst. Health Bull.*, 1935, **164**, 15.

by sealing a membrane of Corning 015 glass to a glass of high electrical resistance, Corning 881, as described by Kahler and Robertson.[4] For use in hamsters and rats, the membrane was 6-8 mm in length and 1 mm in diameter. The internal electrode consisted of a platinum electrode in a saturated solution of quinhydrone in 0.1 *N* hydrochloric acid, which was renewed daily. The electrode was connected to a Beckman pH meter with a shielded cable. The saturated calomel electrode was the asbestos wick type, and made direct contact with the animal so that no additional junction potentials were involved. The whole assembly was kept in a grounded metal box. Measurements were made in an air-conditioned room.

The hamsters were anesthetized intraperitoneally with 8.3 mg of sodium pentobarbital for each 100 g of animal weight. The hamster was laid on its back, and the glass electrode inserted to the full length of the membrane. In the first series of experiments the contact of the reference electrode was just above the teeth on the outside of the mouth. It was, however, found that more uniform readings resulted when the calomel electrode made contact in the roof of the mouth at the base of the teeth. The difference was less than 0.1 pH unit. All readings were made with the temperature compensator set at 35°C. The electrodes were standardized and frequently checked at room temperature with buffers of pH 4.00 and 7.00. The initial pH reading was usually high. It reached an equilibrium value in 5 to 10 minutes, and usually remained constant for about 5 minutes. Prolonged contact gave further changes. These cannot be considered significant as a film was deposited on the electrode from the drying of nasal secretions. This interfered with the uniform wetting of the electrode membrane. After removing the electrode from the nostrils it was washed and checked with the buffers.

To determine the nasal temperature a galvanometer scale was calibrated[5] using a copper-constantan thermocouple in a glass tube similar to those used in the construction of the glass electrode. A period of 5-10 minutes was required to reach an equilibrium value, which corresponded to the time required for the pH measurement to become constant. The hamsters had a nasal temperature of 32-33°C if measured with the thermocouple in the glass tube. Inserting the bare thermocouple directly into the naris gave a value about one degree higher. These values cannot be considered as absolute as the thermocouple cannot be used under conditions identical to those under which it was calibrated.[5] In measuring the pH no difference was observed whether the temperature compensator of the meter was set at 33 or 35°C. The latter setting was used in all determinations.

Results. In the first series 26 pH measurements were made on 10 hamsters. The reference electrode was at the base of the upper teeth on the outside of the mouth. The mean and mean deviation for the pH at 35°C for this series was 7.10 $\pm$ 0.129 with 65% of the values falling within these limits. Another series of 56 determinations in 12 different hamsters (calomel electrode at the base of the teeth in the roof of the mouth) gave a value of pH 7.19 $\pm$ 0.100, with 61% of the readings within these limits. The lower and upper values in this series were 6.93 and 7.42 respectively, with only one reading falling below 7.00. The pH values at 35°C for 3 rats were found to be 7.25, 7.28, and 7.30, and for 4 ferrets, 6.43, 6.75, 6.85, and 7.01. The ferrets were difficult to work with as their movements caused fluctuations in the readings.

The values obtained for hamsters agree well with those of Nungester and Atkinson[2] for the normal pH of the nasal mucosa of man. It is recognized that the readings obtained are not absolute values. Unknown boundary and skin potentials, inability of keeping the whole electrode system at constant temperature, and other errors of the electrode system make it impossible to obtain absolute values at the present time.

In addition to errors involved in making measurements, the Sörensen pH scale cannot at present be precisely defined for solutions other than pure water.[6] Many authors assume

[4] Kahler, H., and Robertson, W. v. B., *J. Nat. Cancer Inst.*, 1943, **3**, 495.

[5] Sheard, C., *Am. J. Clin. Path.*, 1931, **1**, 209.

[6] Nutting, P. G., *J. Franklin Inst.*, 1943, **236**, 573.

that the neutral pH of all solutions is 7.00 at any temperature.[1,2] By definition this is the neutral point only of pure water at 24.9°C. At 35°C $K_w = 2.089 \times 10^{-14}$ [7] so that the neutral pH would be 6.84 for water. The change in pH of buffered solutions with temperature depends upon the buffers present and does not follow the same rules which define the neutral pH. For values near pH 7.0 it is, therefore, better to record the pH as found and the temperature at which the measurement was made without defining its relationship to the neutral point.

Summary. Glass electrodes were constructed for measuring the nasal pH of hamsters, ferrets, and rats *in situ*. The normal pH was found to be 7.2 at 35°C.

[7] Harned, H. S., and Owen, B. B., *Chem. Rev.*, 1939, **25**, 31.

14805

Type Specific Meningococcic Agglutinins in Human Serums. I. Description of Method.

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The presence of meningococcic agglutinins in human serum has been investigated by a number of observers[1] whose studies showed a great variation regarding agglutinin titers and the possible significance of the presence of the antibodies. A difficulty in evaluating these investigations has been the varied technics used in carrying out the agglutination test.

Since April 1, 1942, we have studied serums from 685 individuals for the presence of Group I, Group II, and Group II-alpha agglutinins. These specimens were obtained from the following sources: 276 cases of clinically typical meningococcic infection; 11 family contacts; 17 hospital or laboratory personnel exposures to meningococcic infection; 48 cases of meningitis caused by organisms other than the meningococcus; 102 cases of febrile disease other than meningitis; 46 cases of gonococcic infection;* and 165 unselected serums sent to the laboratory for the Wassermann test.†

Method. The cultures used to prepare the test antigens were standard stock culture strains. These included Group I strains 331 and 1027,‡ Group II strain 36,§ and Group II-alpha strains 1166‡ and 3855.§ Antigens for each of these strains were prepared by washing the 5- to 6-hour growth of well encapsulated meningococci from Miller's medium[2] pint blake bottle slants, heating immediately in a water bath at 65°C for one hour, and diluting to a density of 2×10^{-9} cocci per ml in 0.5% phenolized, buffered saline solution.[3] The diluted antigens were stored

[1] Lingelsheim, W. von, *Klin. Jahnb. Jena*, 1906, **15**, 373; *Z. f. Hyg. u. Infectionskrank.*, 1908, **59**, 457; Elser, W. J., and Huntoon, F. M., *J. Med. Res.*, 1909, **20**, 371; Forster, M., and Gaskell, J. F., *Cerebro-Spinal Fever*, Cambridge, 1916, p. 105; Worster-Drought, C., and Kennedy, A. M., *Cerebro-Spinal Fever*, Black, London, 1919; Lewis, Thomas, Smith, H., and Dingle, J. H., *J. Clin. Invest.*, 1943, **22**, 361.

* Obtained through the courtesy of Miss Agnes C. Hamann of the Diagnostic Laboratory, New York City Department of Health.

† Obtained through the courtesy of Miss Emilie E. Weisse of the Wassermann Laboratory, New York City Department of Health.

‡ Obtained from the National Institute of Health, Bethesda, Maryland, through the courtesy of Dr. Sara E. Branham.

§ Obtained from the Division of Laboratories and Research, New York State Department of Health, Albany, New York, through the courtesy of Miss Sophia M. Cohen.

[2] Miller, C. F., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 989.

[3] Simmons, J. S., and Gentzkow, C. J., *Laboratory Methods of the United States Army*, 1935, 4th Edition, p. 567.

at 5°C. The specificity and agglutinability of each lot of cells was checked by testing them with homologous and heterologous strain rabbit antiserums, and normal human serum. Only such cells were used which were agglutinated by homologous strain rabbit antiserum to titer, and which did not show spontaneous agglutination in 0.85% buffered saline solution, or agglutination in normal human serums in titers exceeding 1-32. Stored cells were tested at bi-weekly intervals to check possible deterioration. Sufficient antigen to last approximately 2 months was prepared in each batch.

Five-tenths ml of serum dilution was placed in 12 x 75 mm test tubes, to which 0.5 ml of antigen was added. Serum dilutions were made in buffered saline solution at pH 7.2. Each serum dilution was increased twofold. The range of final dilutions of 1:8 to 1:512 was modified as the study progressed to 1:50 to 1:1600, so that normal agglutinins would be excluded, and a definite endpoint of the test serum would be obtained.

Methods of Incubation. The standard method of incubation at 37°C for 2 hours, followed by overnight storage at 5°C was the final determination of the serum titer and was used on every serum tested. In addition to this, 2 other methods of incubation were investigated. Six hundred and twenty-eight serums, representative of all the types studied. were centrifuged at high speed for 15 min. immediately after the antigen was added to the serum dilutions.[4] Four hundred and thirty-two (75%) of the tests treated in this way gave identical readings after immediate centrifugation as these same tests did following subsequent incubation at 37°C for 2 hours followed by storage overnight at 5°C. Ninety-one of the remaining 196 (25%) showed slightly higher readings after immediate centrifugation and 105 showed lower readings after immediate centrifugation than they did after incubation and storage in the standard manner. The third method was to delay centrifugation until after the tests had been incubated for 2 hours at 37°C. A group of 414 serums representing all types were tested in this manner. Only 12 (3%) of these showed variation in the readings made after centrifugation and the final overnight reading. The choice of method used for the incubation of the agglutination test was therefore one of expediency.

Reading. Large or medium clumps with a clear supernatant were considered 4+; large or medium clumps in a slightly cloudy fluid 3+; definite clumps in a cloudy fluid 2+; and a few fine clumps in a cloudy fluid 1+. The titer of the test serum was considered as the last tube showing definite clumping (2+). Care had to be taken when the observations were made on the centrifuged tests, since the packing of the cells might appear like clumping when they were first shaken. This was avoided by making a careful comparison with the control suspensions of unsensitized cells. After vigorous shaking, the clumps in the control and negative tubes disappeared, while the clumps in the tubes containing agglutinins persisted, although they became smaller.

Results. The range of normal agglutinins and the specificity of the tests were determined from the results of tests carried out on the first 50 serums of each of (a) the control group of unselected serums sent to the laboratory for the Wassermann test; (b) the group of serums from patients suffering from febrile diseases other than meningococcic infection; and (c) the group of serums from cases of bacteriologically proved meningococcic infection. These results are summarized in Table I. Since none of the serums in the control groups showed the presence of agglutinins in titers above 1-64, agglutinin titers of 1-128 or over were considered significant in indicating the presence of specific antibody, while those up to and including 1-64 were considered within the normal range.

It has been possible to demonstrate agglutinins for one or more of the types of meningococci used in this test in 195 of 276 patients suffering from clinically typical meningococcic infection. In 48 instances it was necessary to test 2 or more specimens of blood at intervals of from 3 to 7 days to obtain agglutinins in significant titers. In fact, failure to demonstrate agglutinins in 72 of the remaining 81 cases may be attributed to the fact that only one serum was tested from 0 to 8 days after

[4] Gates, F. L., *J. Exp. Med.*, 1921, **35**, 63.

TABLE I.

Comparison of Agglutinin Titers in a Control Group of Unselected Serums Sent for the Wassermann Test, a Control Group of Serums Obtained from Patients Suffering Febrile Diseases Other Than Meningococcic Infection, and a Group of Bacteriologically Proved Cases of Meningococcic Infection.

	Serum sent for Wassermann test		Serums from cases of febrile disease other than meningococcic infection		Bacteriologically proved cases of meningococcic infection	
Serum titer	No.	%	No.	%	No.	%
1-512	0	0	0	0	17	34
1-256	0	0	0	0	12	24
1-128	0	0	0	0	6	12
1-64	0	0	6	12	8*	16
1-32	4	8	15	30	4†	8
1-16	17	34	2	4	0	0
No agglutination	29	58	27	54	3†	6
Total	50	100	50	100	50	100

* Six instances only one test serum available.
† Only one test serum available.

onset. No agglutinins were found in the serums of cases of meningitis caused by organisms other than the meningococcus, in the serums of the group of 11 family contacts, or in the serums of 100 of 102 cases of other febrile diseases. It should be noted, however, that agglutinins in significant titers were demonstrated in 6 of 17 healthy hospital or laboratory personnel presumably exposed to meningococcic infection; in 11 of 46 instances of gonococcic infection; in 1 of 2 cases of Rocky Mountain spotted fever; and in 1 of 2 cases of staphylococcic septicemia. The significance of positive findings in cases not due to meningococcic infection merits further investigation.

It is of interest to note that Mayer and Dowling obtained[5] essentially the same results using freshly isolated strains and the centrifuge-agglutination method without further incubation at 37°C.

Summary and Conclusions. The agglutination test, using standardized, type specific, heated and phenolized antigens, prepared from stock culture strains, representative of the prevalent types of meningococci, offers a simple method for the demonstration of agglutinins in the serums of individuals suffering from meningococcic infection. The test may be useful as an additional diagnostic procedure in cases where other laboratory confirmation is lacking.

We are indebted for their cooperation to Misses Ruth Gosling, Helen M. E. Ackerman, and Marie Romano.

[5] Mayer, R. L., and Dowling, H. F., 1944, personal communication.

14806

Size, Hemoglobin Content, and Acid-Soluble Phosphorus of Erythrocytes of Rabbits with Phenylhydrazine-Induced Reticulocytosis.

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Systematic studies of the distribution of acid-soluble phosphorus compounds in the blood cells of various animal species have been under way for some time in our laboratories.[1,2] Present evidence suggests that the distribution may vary with the stage of maturation of the erythrocytes. Kay[3] reported observations on the distribution of acid-soluble P in the bloods of patients with splenic anemia, acholuric jaundice, hemolytic icterus during crises, and pernicious anemia during treatment with liver extract. Such bloods, which are characterized by increased percentages of reticulocytes, exhibited relatively high cellular concentrations of organic acid-soluble P. There was also indirect evidence that nucleoprotein, generally believed to be absent in mature erythrocytes, was present in these bloods in amounts roughly proportional to the reticulocyte count.

In order to elucidate the suggested relationship further we have now determined the distribution of acid-soluble P in the bloods of rabbits following repeated injections of phenylhydrazine which, as reported by Watson,[4] leads to the appearance of large numbers of reticulocytes in the blood. In the bloods of the rabbits so treated, estimations were made of the inorganic P, total acid-soluble P, adenosine triphosphate P, and in some instances the diphosphoglycerate as well as of the dimensions and of the hemoglobin content of the cells. Measurements of the osmotic fragility of the cells, which were made at the same time, will be described in a later communication.

Methods. The methods used for the collection of blood and for the determination of the various phosphorus fractions have been described previously.[1] Reticulocyte counts were made on wet preparations. A drop of blood was spread on a slide under a coverslip on which a film of brilliant-cresyl-blue had been dried. The number of reticulocytes seen during the counting of 500 erythrocytes was noted; if the percentage of reticulocytes was low, 1000 erythrocytes were counted. Two pipettes and 2 hemocytometers, certified by the Bureau of Standards, were used. Ten large squares in each of the chambers—a total of 40 large squares (0.16 mm^3)—were counted.

The volume of packed cells was determined by the method of Guest and Siler.[5] The hemoglobin was measured in an Evelyn colorimeter, using filter 540.

Experimental. Full grown white New Zealand rabbits weighing 2.5 to 3.5 kg were used. To produce reticulocytosis a 3.3% solution of phenylhydrazine hydrochloride, adjusted to pH 7 by the addition of NaOH, was administered subcutaneously. As a rule the phenylhydrazine was injected according to the following schedule: first day, 33 mg; second day, 66 mg; fourth day, 33 mg; and from then on, 33 mg every second day. The total dose given and the intervals between the injections were varied in individual animals according to the degree of anemia that developed and the losses of weight observed from day to day. Usually, in the successful experiments, the animals lost little weight. Among the rabbits so treated the mortality was fairly high during the first 4 days, but few of the animals surviving longer periods of time died. Blood samples for chemical analyses were drawn before the first injection and at varying intervals during the

1 Guest, G. M., and Rapoport, S., *Physiol. Rev.*, 1941, **21**, 410.

2 Rapoport, S., and Guest, G. M., *J. Biol. Chem.*, 1941, **138**, 269.

3 Kay, H. D., *Brit. J. Exp. Path.*, 1930, **11**, 148.

4 Watson, C. J., and Clarke, W. O., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 65.

5 Guest, G. M., and Siler, V. E., *J. Lab. and Clin. Med.*, 1934, **19**, 757.

TABLE I.

Size, Hemoglobin Content, and Acid-Soluble P in Erythrocytes of Rabbits Treated with Phenylhydrazine.

Animal No.	Vol. packed cells, %	Rbc count, mill.	Mean cell vol., μ^3	Hb conc. cells, g/100 cc	Mean Hb per cell, γγ	Reticulocytes		ATP cells, mg/100 cc	OASP cells, mg/100 cc	Notes	
						Total, %	Heavy, %				
C 338	41.1	6.34	65	33.8	21.9	2	0	14.1	73.6	Before treatment	
	17.7	2.45	72	29.0	21.0	88	27	18.7	79.6	4th day "	
	32.7	2.42	135	23.6	31.8	61	40	23.3	83.0	8th " "	
	25.5	2.03	126	22.3	28.1	99	85	31.9	94.3	12th " "	
	19.2	1.28	150	20.9	31.3	99	88	29.7	86.5	15th " "	sacrificed
C 339	46.6	7.46	63	34.0	21.5	2	0	13.5	81.5	Before treatment	
	21.4	3.46	62	32.7	20.2	96	15	17.0	78.4	3rd day "	
	24.0	2.29	105	27.6	29.1	79	61	30.3	98.3	6th " "	
	25.1	2.16	117	25.0	29.1	95	53	27.5	99.7	8th " "	
	21.2	1.94	109	24.2	26.4	98	80	31.2	108.9	10th " "	ended
	32.4	3.23	100	25.1	25.2	46	31	23.5	100.8	8th " recovery	
	37.9	4.54	84	27.1	22.7	22	15	13.2	88.8	12th " "	
	41.4	5.74	72	29.3	21.2	2	0	12.8	—	18th " "	
C 337	37.2	5.93	63	33.8	21.2	3	0	9.4	75.9	Before treatment	
	14.9	1.51	99	27.7	27.4	82	39	25.5	91.2	9th day "	
	18.5	1.65	112	26.9	30.2	97	74	29.2	100.8	13th " "	ended
	29.8	2.85	105	25.9	27.1	45	29	20.0	94.6	3rd " recovery	
C 323	42.4	7.05	60	34.4	20.7	2	0	15.2	80.2	Before treatment	
	20.4	3.50	58	36.4	21.2	86	16	14.3	75.8	3rd day "	
	17.4	1.25	139	23.9	33.1	99	84	33.5	102.4	6th " "	
C 322	19.3	1.50	129	22.3	28.7	72	—	31.4	101.4	9th day treatment	
	23.1	1.85	125	23.3	29.1	79	—	35.8	105.1	10th " "	ended
	27.9	2.86	98	26.0	25.4	42	—	20.8	78.2	4th " recovery	
	41.9	6.70	63	32.3	20.2	2	—	14.0	79.6	12th " "	

course of injections and the period of recovery. Small blood samples for determinations of the reticulocyte count, total red cell count, and hemoglobin content were taken daily during the period of injections, but less frequently after the injections were stopped. By the fourth day a high percentage of the erythrocytes appeared to contain reticulum, and many crenated cells were seen. At first the reticulum was very fine and in some instances difficult to distinguish from crenation. During this time the mean cell volume was little if any increased. Around the sixth to eighth day, nearly 100% of the erythrocytes showed heavy reticulation, differing markedly from that found on the fourth day, and a high mean cell volume was attained, sometimes exceeding twice the initial normal value. In some rabbits it was possible to maintain close to 100% reticulocytosis for more than a week by repeated injections of small doses of phenylhydrazine.

Results and Discussion. In Table I are cited data on the distribution of the acid-soluble P, changes in dimensions and hemoglobin content, and the percentage of reticulocytes in the bloods of several rabbits following the administration of phenylhydrazine. It can be seen that the volume of the packed cells decreased in each animal to ½ or less of the normal value within 3 days after the start of injections, but that further change during longer periods of treatment was slight. The red cell count on the other hand continued to decrease while phenylhydrazine was administered. The mean cell volume (MCV) of the cells seemed to increase in proportion to the number of heavily reticulated cells, reaching values of 150 μ^3, about 2.5 times the normal value. These large cells contain somewhat more hemoglobin per cell than mature erythrocytes, but the corpuscular concentration of hemoglobin decreased markedly, to values about ⅔ of normal. The increases of adenosine triphosphate (ATP) in each instance account for a large part of the increased concentration of organic acid-soluble P (OASP) in the cells. The adenosine triphosphate reached a maximum concentration of approximately 30 mg per 100 cc of cells, but did not increase in concentration further despite continued injections of the phenylhydrazine. The

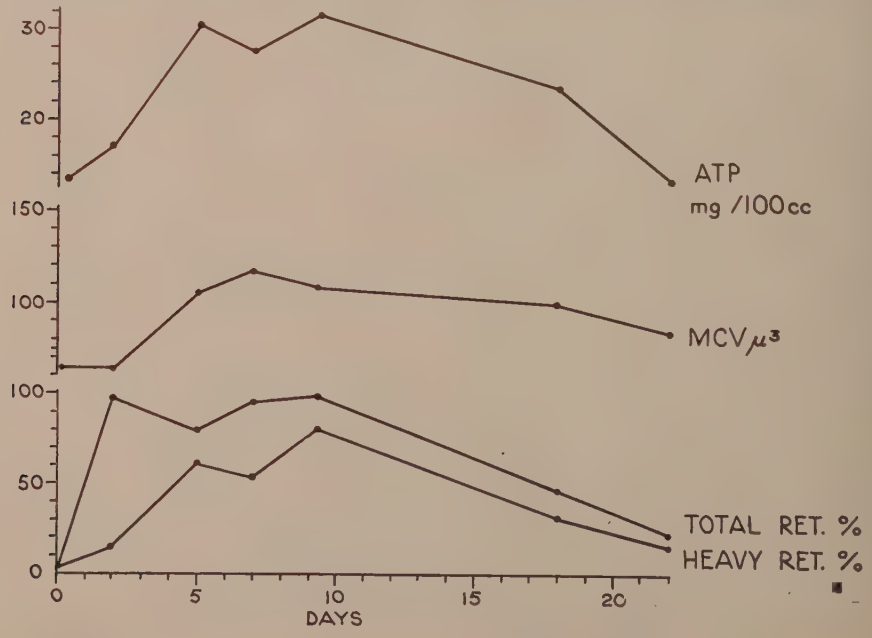

FIG. I

RABBIT C 339

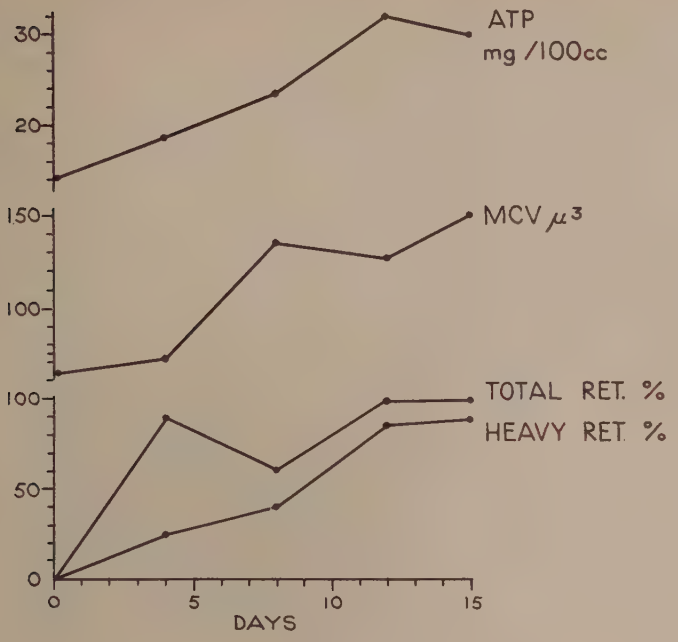

FIG. II RABBIT C 338

concentration of the diphosphoglycerate fraction, determined in some of the samples, did not change significantly.

The charts indicate more clearly than the table a correlation between the changes in the concentration of adenosine triphosphate and the variations in the mean cell size and the proportion of reticulocytes. Two curves portray changes in the reticulocytes, one depicting the proportion of all reticulated cells, the other representing the proportion of coarsely reticulated cells. The curves indicate that the finely reticulated cells are smaller in size than the coarsely reticulated cells, and contain less adenosine triphosphate. In the blood of the animal represented in Chart 1, on the second day after the start of the injections, 96% of the cells were reticulated, most of them finely, but neither the mean cell size nor the adenosine triphosphate content were materially changed; on the ninth day, the proportion of the reticulocytes was about the same as on the second day, but a great majority of the cells showed coarse reticulation and both mean cell size and the adenosine triphosphate content had increased to about twice the initial value. Chart 2 likewise suggests a close relationship between the mean cell size, adenosine triphosphate content, and the number of coarsely reticulated cells. Such evidence suggests the existence of an immature cell, the coarsely reticulated erythrocyte, twice or more the size of the normocyte, containing a high concentration of adenosine triphosphate and a lower concentration of hemoglobin.

The origin and significance of the increased concentration of adenosine triphosphate in the reticulocytes are unknown. Possible relationships between the altered distribution of phosphorus compounds and metabolism of these cells have been discussed earlier.[2]

Summary. Following repeated injections of phenylhydrazine in rabbits, varying degrees of reticulocytosis, up to 100%, occur. With the increasing reticulocytosis the concentration of adenosine triphosphate in the erythrocytes increases from initial concentrations of 10 to 15 mg to 30 mg or more per 100 cc. The mean volume of the cells is greatly increased from around 60 μ^3 up to 150 μ^3, and the corpuscular hemoglobin concentration is decreased from 34 to 22 g per 100 cc of cells.

14807

Studies *in Vitro* on Physiology of Cells: Effect of Anisotonic Solutions.*

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The effect of osmotic pressure on erythrocytes has been extensively studied partly due to the intrinsic importance of the red blood cells and partly due to the ease in obtaining a suspension of the cells, in observing their hemolysis and in examining them microscopically by smears or in suspension. As a result of these studies, considerable information has been obtained on the effect of anisotonic solutions on erythrocytes and on the permeability of the cell membrane to various reagents.[1]

The method of unstained cell counts[2] permits analogous studies using cells in suspensions derived from the thymus, testis, and other organs.

Effect of Distilled Water. The methods used for preparing cellular suspensions and for counting the number of viable cells in the suspensions are presented in detail in previous papers.[2] In brief, the thymus or other organ is chopped up with scissors after adding a small amount of Ringer's solution which was buffered with 20% of Sorensen's phosphate solution having a pH of 7.7. This solution is isotonic with the cells and did not affect the size or shape of red blood cells. The cells in the suspension were washed with and then suspended in the phosphate-Ringer solution. To 0.2 ml amounts of suspension were added 0.1 to 0.8 ml of distilled water. The mixtures were shaken 15 minutes in a water bath at 37.5°C. After incubation, a solution of eosin (1:1000 in Tyrode's fluid) was added to make a final volume of 4.0 ml. The presence and character of any precipitate were noted and counts were made of the stained and unstained cells and of the red blood cells. Earlier papers[2] have demonstrated that the unstained cells are, in all probability, viable and that the stained cells are dead. Suspensions of erythrocytes were diluted with Tyrode's solution instead of eosin and the amount of hemolysis was determined after centrifugation. The results are shown in Table I.

The macroscopic observations on the effect of distilled water on cellular suspensions were of considerable interest. Distilled water caused hemolysis in suspensions of erythrocytes, 0.1 ml of water producing partial and 0.4 ml complete hemolysis. No gross changes were observed on the addition of water to suspensions of polymorphonuclear leukocytes and splenic cells. Testicular cells in suspension were clumped by distilled water. The white, opaque clumps were readily broken up by drawing them back and forth in a pipette and the cells were resuspended. On the other hand, the addition of distilled water to suspensions of thymic cells of the rat or rabbit resulted in the precipitation of a large, gelatinous, translucent mass which was not dissolved by the addition of the eosin solution. The greater the number of thymic cells in suspension, the larger was the precipitate. The supernatant was clear when large amounts of water were added (0.4 and 0.8 ml) and was turbid after the addition of smaller volumes of water (0.1 and 0.2 ml).

A few experiments were performed to study the nature of the characteristic precipitate produced in thymic suspensions. The addition of isotonic solutions of sucrose instead of distilled water did not cause precipitation. This finding indicates that the action of distilled water was not due to the dilution of the electrolytes but was rather the effect of the reduced osmotic tension. The addition of distilled water to thymic cells killed by heating at 55°C for one hour also resulted in a pre-

* Published with the permission of the Medical Director of the Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the author.

1 Cowdry, E. V., *General Cytology*, Chicago, University of Chicago Press, 1924.

2 Schrek, R., *Am. J. Cancer*, 1936, **28**, 389; *Arch. Path.*, 1943, **35**, 857; *ibid.*, 1944, **37**, 319; PROC. SOC. EXP. BIOL. AND MED., 1944, **54**, 283.

TABLE I. Toxic, Hemolytic, and Precipitating Action of Distilled Water on Cells in Suspension.

Ml distilled water added to 0.2 ml of suspension	% distilled water in final susp.	Red blood cells of rabbit	Thymus of rat	Thymus of rabbit	Spleen of rat	Lymphatic leukemia of mouse	Lymphatic leukemia of man	Peritoneal exudate of rat	Testicle of rat
Macroscopic observations									
0	0		T	T	T	T	T	T	T
0.1	33	H 2+	T	T		T	T		T
0.2	50	H 2+	P 1+	P 1+		T	T	T	T
0.4	67	H 3+	P 2+	P 1+	T	T	C 1+	T	C 1+
0.8	80	H 3+	P 2+	P 2+	T	C 1+	C 1+	T	C 1+
No. of unstained cells per millimicroliter									
0	0	265	112	110	356	206	181	87	158
0.1	33	80	110	93		195	207		65
0.2	50	35	101*	25*		182	225	91	38
0.4	67	6	49*	3*	195	87	186	31	28
0.8	80	0	7*	1*	70	**28**	90	9	13

H = Hemolysis.
T = Homogeneously turbid.
P = Gelatinous precipitate which cannot be resuspended.
C = Clumping of cells which can be broken up.
1+ = Slight degree.
2+ = Moderate degree.
3+ = Marked degree.
* Count is unreliable due to the presence of precipitate.

cipitate which was, however, somewhat opaque instead of translucent and was not as voluminous as in the unheated suspension. In one experiment the mass was washed with phosphate-Ringer solution and then chopped up with scissors and filtered through wire gauze. The resultant suspension was found to contain a small number of cells, some of which were still viable. The precipitate, on histologic section, consisted of a network of thin basophilic fibrils. Slightly enlarged and normal-sized cells, in moderate numbers, were attached to the fibrils. The nuclei of the cells stained deeply and some were indented markedly so that they appeared in the shape of a horseshoe or ring.

The toxicity of distilled water was determined by counting the number of erythrocytes or viable cells (*i.e.*, cells resistant to staining with eosin) after 15 minutes exposure to the water. Table I shows that the cells differed in their susceptibility to the toxic action of distilled water. The addition of 0.2 ml of distilled water to 0.2 ml of suspension resulted in (1) a marked reduction in the number of red blood cells (from 265 to 35), (2) a considerable decrease in the number of viable testicular cells (from 158 to 38), and no appreciable change in the number of polymorphonuclear leukocytes, leukemic cells, and thymic cells of the rat. It was observed, furthermore, that testicular and other cells were lysed by high concentrations of distilled water so that the total numbers of stained and unstained cells in the suspensions were decreased. It may be concluded that distilled water had a more deleterious effect on erythrocytes and testicular cells than on the other cells tested.

Effect of Freezing and Thawing. Experiments on the freezing of cellular suspensions gave results which were to some extent similar to those obtained on the addition of distilled water. A suspension of thymic cells was frozen with solid carbon dioxide for 10 minutes and then thawed in the water bath at 37.5°C. As a result of this procedure, the suspension developed a large, white gelatinous precipitate. A histologic section of the precipitate showed parallel strands of basophilic fibrils to which were attached a moderate

TABLE II. Toxic Effect of Sodium Chloride, Sucrose, Ethyl Alcohol, and Urea in Varying Concentrations.

Solvent	Molarity of reagent	Number of unstained cells per millimicroliter: Thymus of rat: Sodium chloride	Thymus of rat: Sucrose	Thymus of rat: Urea	Thymus of rat: Ethyl alcohol	Testicle of rat: Sodium chloride	Testicle of rat: Sucrose	Testicle of rat: Urea	Testicle of rat: Ethyl alcohol	Peritoneal cells: Urea
Distilled water	0			5*				9		
	0.02	14*				12				
	0.05	53*	65*			20	11			
	0.1	109	100*	7*	12*	159	21	11	25	
	0.2	97	110	11*	12*	153	57	19	27	
	0.5	104	117	10*	18*	85	45	5	25	
	1.	39†	113	7	7*	60	59	3	17	
	2.	2†	11	1	0	51†	44	1	9	
Phosphate-Ringer	0	115	139	230	208	154	83	220	173	108
	0.1			200				177		
	0.2			223	167			72	184	
	0.5			256	188			22	130	92
	1.			201	194			7	221	97
	2.			155	162			4	196	31

* Count is not reliable due to precipitate. † Cells are clumped but can be readily resuspended.

number of lymphocytes. Under identical conditions, suspensions of splenic and testicular cells failed to develop any precipitate. Freezing and thawing, like distilled water, caused a precipitate in thymic but not in the other suspensions.

A few incidental observations were made in the above experiments. The freezing and thawing caused the lysis of the erythrocytes and the death of most of the nucleated cells. However, an occasional lymphocytic and testicular cell remained viable as indicated by its resistance to staining with eosin.

Effect of Sodium Chloride and Sucrose. Various concentrations of sodium chloride and of sucrose in distilled water and in phosphate-Ringer solution were added to cellular suspensions (0.8 ml of reagent to 0.2 ml of suspension). The mixtures were shaken for 15 minutes at 37.5°C and then examined.

It is seen from Table II that sodium chloride and sucrose were relatively non-toxic at certain concentrations (0.1 to 0.5 molar for sodium chloride and 0.2 to 1.0 molar for sucrose) which are isotonic or moderately anisotonic for the cell. The method was not suitable for determining the concentration of isotonic solutions since fluids which were slightly or moderately anisotonic were not lethal to the cell.

Markedly hypotonic solutions of sodium chloride and sucrose decreased the number of viable cells and also caused a gelatinous precipitate in suspensions derived from the thymus. These findings are in accord with those observed on the addition of distilled water.

Markedly hypertonic solutions of the 2 reagents were also found to be toxic to testicular and other cells. The hypertonic solutions of sodium chloride caused clumping of the cells. The clumps could be broken up by drawing them back and forth in a pipette.

Effect of Ethyl Alcohol and Urea. It has been shown that sodium chloride and sucrose in distilled water were relatively non-toxic in certain concentrations. In contrast ethyl alcohol in distilled water was found to be cytocidal at all the concentrations studied (0.1 to 2 molar). It was observed furthermore that alcohol in distilled water caused the same type of gelatinous precipitate in thymic sus-

pensions as was observed on the addition of distilled water alone. On the other hand, ethyl alcohol dissolved in phosphate-Ringer solution had little or no lethal effect on thymic and testicular cells even in high concentrations (2 molar). A 3-molar solution had a marked cytocidal action in fifteen minutes at 37.5°C.

From these experiments, it seems that ethyl alcohol exerts little if any osmotic tension on the cells. This would indicate that the cell wall was readily permeable to the reagent. In spite of its apparent capacity to penetrate into the cell, ethyl alcohol has only a slight cytocidal action.

Similar experiments with urea showed that this reagent also failed to exert an osmotic pressure on the cell. Evidently the cellular wall was also permeable to this compound. Urea in 1 molar solution had no appreciable effect on the number of viable thymic cells or polymorphonuclear leukocytes and did not cause the hemolysis of erythrocytes but did cause a marked decrease in the number of viable testicular cells (from 220 to 7 cells per millimicroliter). Evidently urea had a marked toxic effect on testicular cells but not on the other cells studied.

Summary. By means of the method of unstained cell counts both distilled water and urea were found to be more toxic to testicular cells than to lymphocytes and polymorphonuclear leukocytes. Ethyl alcohol was not cytocidal even in relatively high concentrations. The cell membrane of viable thymic, testicular and other cells appeared readily permeable to urea and ethyl alcohol but not to sodium chloride or sucrose. The addition of distilled water or freezing and thawing produced a characteristic gelatinous precipitate only in suspensions of thymic cells but not in suspensions of splenic or testicular cells.

14808

Effect of Penicillin on Certain Viruses.

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In view of the wide antibiotic activity of penicillin, it is of interest to learn whether it has any effect on the growth of viruses. The present paper deals with experiments designed to gain information on this subject.

Certain representative viruses were used as follows: Vaccinia BH,[1] a strain of vaccine virus derived originally from the New York Board of Health Strain and maintained for several years by rabbit testicular passage; Vaccinia CV II,[1] derived from BH by serial passage in Rivers-Li culture medium now of apparently stable virulence; St. Louis Encephalitis, Hubbard strain, obtained from Dr. Margaret E. Smith, and maintained by serial intracerebral mouse passage; Psittacosis 6 BC, obtained through the courtesy of Dr. J. E. Smadel, maintained by serial yolk sac passages; Meningo-pneumonitis virus, M.P., obtained from Dr. T. Francis, Jr., and maintained by mouse lung and chick embryo yolk sac passage; Equine Encephalomyelitis virus, the Eastern strain, secured through the courtesy of Dr. J. E. Beard, was used in its third chick embryo passage.

Penicillin used was prepared by the Reichel and Abbott Laboratories and was part of a supply allocated to the University Hospitals of Cleveland by the Committee on Therapeutics and Other Agents of the National Research Council. Assays were made by the Oxford cup method using spores of *Bacillus subtilis* for inoculating the solid medium as suggested by Foster and Woodruff.[2] It was found that

[1] Parker, R. F., Bronson, L. H., and Green, R. H., *J. Exp. Med.*, 1941, **74**, 263.

[2] Foster, J. W., and Woodruff, H. B., *J. Bact.*, 1944, **47**, 43.

a much greater yield of spores could be obtained under conditions of partial desiccation of the culture than in the liquid medium originally proposed. The culture was accordingly seeded on the surface of nutrient agar, and incubated for 7 days. It was then harvested and pasteurized and the spore suspension stored at 4°C. If assays were not carried out immediately, the sample was stored at −15°C. Appropriate controls were always included.

Two general methods were used for testing the effect of penicillin on virus growth: (1) cultivation in Rivers-Li culture medium[3] consisting of chick embryonic tissue suspended in Tyrode solution, in a total volume of 10 ml; and (2) cultivation in the intact chick embryo. When the first method was employed, a measured quantity of virus was added to the culture medium, titration of an aliquot portion being carried out as soon as possible after setting up the culture. After 5 days' incubation 0.1 ml of the culture was transferred to fresh medium, giving a dilution for each passage of 10^{-2}. The culture after incubation was titrated for virus and a penicillin assay was made. When the intact chick embryo was used, inoculation was made into the yolk sac[4] or onto the chorio-allantoic membrane.[5] Death of the embryo or development of lesions on the membrane was taken as the criterion of virus growth, appropriate bacteriologic controls being included.

Since the time of incubation or time to death of the embryo would for most of the viruses be measured in days, it was necessary to gain information on the rate of deterioration of the penicillin. Accordingly several flasks of the culture medium were set up and penicillin was added, but no virus. They were incubated at 37°C. A sample was removed each day from one of the flasks, stored at −15°C, and finally all samples were collected and titrated by the cup method together with controls. Data obtained in such an experiment are presented in Fig. 1. The deterioration of the substance is seen to be regular over the whole period of observation. A number of assays were made on cultures seeded with virus, and the results of 8 of these are included in the figure. Similar data were obtained on the stability of penicillin when injected into the chick embryo, and are presented in Fig. 2. In these experiments penicillin was injected into the yolk sac

Fig. 1.

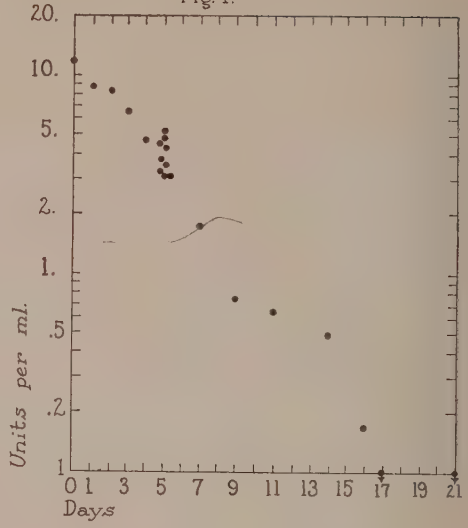
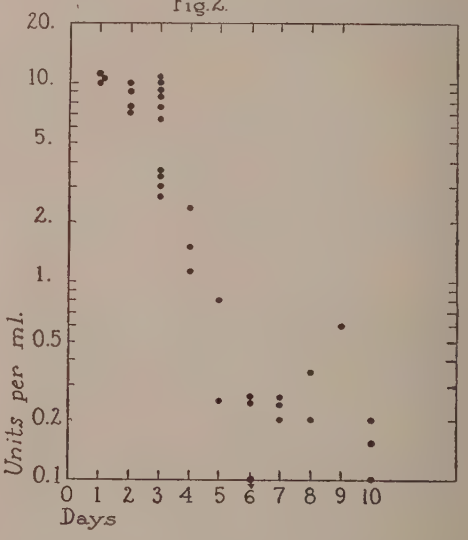


Fig. 2.



[3] Li, C. P., and Rivers, T. M., *J. Exp. Med.*, 1930, **52**, 465.

[4] Cox, H. R., *U. S. Pub. Health Rep.*, 1938, **53**, 2241.

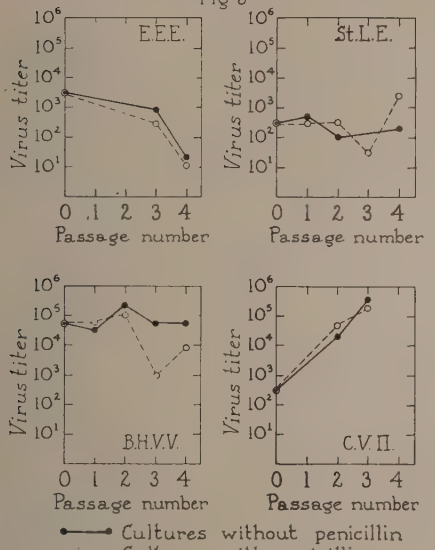
[5] Barret, F. M., *J. Path. and Bact.*, 1933, **37**, 107; Woodruff, A. M., and Goodpasture, E. W., *Am. J. Path.*, 1931, **7**, 209.

TABLE I.
Virus-Penicillin Experiments with Intact Chick Embryos.

Days after injection of virus and penicillin	M.P.V.*		M.P.V.†		6 B.C.‡		6 B.C.§	
	Controls	Penicillin	Controls	Penicillin	Controls	Penicillin	Controls	Penicillin
0	0	0	0	0	0	0	0	0
1	2‖	0	2	1	1	0	0	0
2	1	1	2	1	1	0	0	1
3	0	0	0	2	1	0	0	0
4	0	0	0	1	2	0	0	2
5	0	1	0	0	8	4	1	0
6	0	0	2	1	13	0	14	4
7	3	0	7	1	1	2	3	1
8	11	0	4	1	1	1	0	3
9	0	1	2	1	0	3	0	2
10	2	0	1	1	1	9	0	2
11	0	2		0		1	0	1
12	0	1		4		7	0	4
13	0	0		5			0	4
Survivors at 14th day	1	24	0	11	0	2	2	5

* Meningo-pneumonitis virus. $10^{3.2}$ infectious units injected per egg.
Penicillin: 200 U per egg injected initially + 200 U on 4th day.
† Meningo-pneumonitis virus. $10^{3.2}$ infectious units injected per egg.
Penicillin: 50 U per egg injected initially + 50 U on 3rd day.
‡ Psittacosis virus. $10^{2.5}$ infectious units injected per egg.
Penicillin: 500 U per egg initially.
§ Psittacosis virus. $10^{2.5}$ infectious units injected per egg.
Penicillin: 666 U per egg initially + 600 U on 3rd day.
‖ Figures indicate number of embryos dying on indicated day.

Fig 3



of embryos of 5 days' incubation. The eggs were opened at intervals, the fluid mixed with a pipette and a sample withdrawn. Before assay, the fluid was centrifuged to remove red cells and debris which interfered with the diffusion of penicillin. Progressive desiccation of the embryo prevented an indefinite continuance of the assays. It is seen that as with the liquid cultures, penicillin disappeared slowly.

The results of the experiments with viruses cultivated in the chick-embryo medium are given in Fig. 3. The first 2 passages of BH Vaccinia and St. Louis Encephalitis were in medium containing 1 unit of penicillin per ml at the beginning of incubation. Later passages, and all passages of CV II Vaccinia and Equine Encephalomyelitis were in medium containing 10 units per ml initially. Samples taken from these flasks after 5 days' incubation contained from 3.0 to 5.2 units of penicillin per ml. It will be seen that the titration values for the control do not differ significantly from values for the "penicillin" cultures.

In one series of experiments, the 2 strains of vaccinia were inoculated on the chorio-allantois of 14-day embryos. A quantity of virus was used which gave 15 to 50 lesions on each membrane. In one series 500 units of penicillin were injected into the egg on the day before inoculation of virus, and in the

TABLE II.
Effect of Penicillin on Equine-Encephalomyelitis in Chick Embryos.

Dilution of virus	Control	Treated
10^{-3}	8/8	7/7
10^{-4}	18/18	14/15
10^{-5}	8/8	6/8

Numerator: number dying; denominator: number inoculated.
Titer of virus in previous experiment: 10^{-6}.
500 units penicillin injected into each egg just before virus inoculation.

other, 500 units of penicillin were mixed with the virus inoculum. In neither case could any difference between control and treated embryos be distinguished, either as to size or number of lesions when the membranes were examined at 48 hours.

The results of inoculation of chick embryos with psittacosis and with M.P. virus, and with penicillin plus these viruses, are given in Table I. It will be seen that with these 2 infections penicillin was effective in delaying, or with larger doses preventing death of the embryos within the period of observation. Not all embryos dying were tested for the presence of virus but enough passages were made to learn that in a fair proportion of the embryos dying late after penicillin treatment, no detectable virus was present.

In one series of experiments embryos were inoculated with equine encephalomyelitis virus, and the results are recorded in Table II. The penicillin was entirely without effect on the course of the infection.

It is evident from these experiments that penicillin in the doses used was without effect on the multiplication of the viruses of vaccinia, St. Louis encephalitis or equine encephalomyelitis, or on the course of the disease induced in chick embryos by vaccinia or equine encephalomyelitis. Penicillin did have a very definite effect on the course of the disease induced in chick embryos by the viruses of psittacosis and of meningo-pneumonitis. It is to be noted, however, that the doses used were large. To maintain a comparable blood concentration of the drug would require the injection into man of several million units of penicillin daily. The practical importance of this observation may, therefore, be questioned.

14809

Isolation of Influenza A Virus from Normal Human Contacts During an Epidemic of Influenza A.*

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Various investigators have reported the occurrence of sub-clinical infections during epidemics of influenza A as evidenced by significant increases in neutralizing or complement fixing antibodies in the sera of persons who were exposed but who did not become ill.[1,2,3] A review of the literature, however, has revealed no reports of the isolation of influenza virus from persons with subclinical infections following normal exposure to the disease.

In November, 1943, 171 typical clinical cases of influenza A occurred in a dormitory housing approximately 500 students at the

* This study was supported by funds from the International Health Division of the Rockefeller Foundation.

1 Francis, T., Jr., Magill, T. P., Rickard, E. R., and Beck, M. D., *Am. J. Pub. Health*, 1937, **27**, 1141.

2 Stuart-Harris, C. H., *et al.*, *Medical Research Council, Special Report Series*, No. 228, 1938.

3 Rickard, E. R., Lennette, E. H., and Horsfall, F. L., Jr., *Pub. Health Rep.*, 1940, **55**, 2146.

University of Minnesota. Provision for the medical care of the students was such that there was little probability that any individual suffering from the usual symptoms of influenza would escape observation. In order to study the incidence of subclinical infections 14 apparently normal students living in the dormitory were questioned in detail as to symptoms suggestive of influenza. None of these men considered himself to be ill and the few symptoms which were elicited were of such trivial nature as might be expected to be obtained by questioning any group of apparently normal individuals during the winter season.

From 13 of the 14 men naso-pharyngeal washings were taken at the time of questioning. A few cc of 20% normal horse serum-broth were instilled into the nostrils. After expectorating, the subject gargled with a small quantity of the same menstruum and expectorated into the container holding the nasal washings. The combined washings from the nose and throat were placed immediately on CO_2 ice where they were kept until examined. Blood specimens were obtained from 13 of the 14 men at the time the throat washings were taken and again at approximately two weeks later.

Materials and Methods. Throat washings were examined for the presence of influenza virus by the intra-allantoic inoculation of 11-day embryonated eggs with unfiltered washings.[4] The avian red blood cell agglutination phenomenon[5] was used as an indicator of the presence of virus. A minimum of 5 serial passages was performed with each washing before considering it negative.

Viruses isolated were identified as influenza A by comparing the red blood cell agglutination inhibition[6] titer of a pool of acute phase human anti-influenza A serum to the titer of a pool of convalescent phase serum from the same subjects. In every instance when these pools were tested against the unknown strains, there was an increase of at least 4 times in the titer of the convalescent pool as compared to the acute phase pool. The average increase in titer against the 5 strains was 14 times.

Serum specimens were examined by the red blood cell agglutination inhibition test and end points were determined by the use of the photo-densitometer.[7] In all instances pre- and post-infection sera were examined in the same test. The antigens employed were 4 minimal agglutinating doses of the PR8[8] strain of influenza A virus and the Lee[9] strain of influenza B virus, both egg-adapted and contained in infected allantoic fluids.

Results. Details of virus isolation and serum examination have been summarized in Table I. It may be noted that 9 of the 13, or 69%, of the pairs of sera examined showed significant, or greater than fourfold, increases in antibodies to influenza A. None of the sera was positive to influenza B. Influenza A virus was isolated from 5 of the 13, or 38%, of the throat washings examined.

These percentages of positive virus isolation and antibody response were very similar to those obtained in the examination of material taken from clinical cases of influenza which occurred in the same dormitory. From the clinical cases 86 throat washings were examined and influenza A virus was isolated from 32, or 37%, of the specimens. Of 71 pairs of acute and convalescent serum specimens examined from the clinical cases in 61, or 86%, significant increases in antibodies for influenza A virus were observed.

Discussion. Of considerable interest is the finding that in the sera of three individuals from whose throat washings virus was isolated no significant increase in antibodies was noted. Although the importance of subclinical infection in the immunization to and the dissemination of influenza has long been appreciated, the observations herein recorded further emphasize the necessity of considering this factor in attempting to control the spread of the disease. The ease with which virus was isolated from the throats of apparently normal subjects suggests that such persons may be of equal, if not greater, importance in spread-

[4] Rickard, E. R., Thigpen, M. P., and Crowley, J. H., *J. Immunol.*, 1944, **49**, 263.

[5] Hirst, G. K., *Science*, 1941, **94**, 22.

[6] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

[7] Hirst, G. K., and Pickels, D. G., *J. Immunol.*, 1942, **45**, 273.

[8] Francis, T., Jr., *Science*, 1934, **80**, 457.

[9] Francis, T., Jr., *Science*, 1940, **92**, 405.

TABLE I.
Serological Findings and Virus Isolation from Apparently Normal Contacts.

Case No.	Serological diagnosis influenza A: Acute phase titer	Convalescent phase titer	Significant increase antibodies	Virus isolation Egg passage in which agglutination was first observed	Symptoms
1	1:208	1:1450	+	Fourth	Feeling onset of cold
2	1:223	1:1270	+	Neg.	Slight cough
3	*	*	*	Third	Slight coryza
4	1:91	1:315	+	Neg.	Chronic cough
5	1:147	1:16,400	+	Neg.	No symptoms
6	1:447	1:722	—	Fourth	,, ,,
7	1:91	1:2900	+	Neg.	Slight sore throat
8	1:84	1:97	—	Third	Chronic cough
9	1:21	1:356	+	Neg.	Slight cough
10	1:362	1:891	—	Neg.	No symptoms
11	1:132	1:722	+	Neg.	,, ,,
12	1:223	1:1024	+	*	,, ,,
13	1:223	1:1270	+	Neg.	Slight coryza
14	1:223	1:512	—	Third	No symptoms

* Not examined.

ing infection than are the clinical cases.

Summary. By the direct intra-allantoic inoculation of unfiltered throat washings influenza A virus was isolated from 5 of 13 apparently normal contacts during an epidemic of influenza A. Significant increases in neutralizing antibodies were demonstrated in the sera of 9 of 13 such contacts by means of the red blood cell agglutination inhibition test.

14810

Agglutination Tests with Streptococcus No. 344 in Primary Atypical Pneumonia.*

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The discovery by Thomas, Mirick, Ziegler, Curnen, and Horsfall[1] that sera of patients convalescent from primary atypical pneumonia frequently agglutinate an indifferent streptococcus, labeled No. 344, has provided yet another method for investigating this disease. The fact that the streptococcus agglutinins persist for a long time when stored at 4°C has made it possible to investigate sera from patients with primary atypical pneumonia received at this laboratory since the summer of 1941. Results of the tests, together with certain observations correlating laboratory and clinical findings, form the subject of this report.

Methods and Materials. The method described in the original report was followed without modification. Titers of 10 or more were considered significant.

Sera were collected from students admitted to Cowell Memorial Hospital, University of California, Berkeley, and to the University of

* The studies and observations on which this paper is based were supported by the International Health Division of The Rockefeller Foundation in cooperation with the California State Department of Public Health.

[1] Thomas, L., Mirick, G. S., Curnen, E. C., Ziegler, J. E., and Horsfall, F. L., Jr., *Science*, 1943, **98**, 566.

California Hospital, San Francisco; from patients admitted to certain west coast military establishments; and from scattered patients in the San Francisco area. Both early and late specimens were available in most cases. Those specimens taken during the first week of illness were considered acute. Convalescent specimens were collected between the twelfth and forty-fifth days.

Clinical Observations. Cases were classified prior to this investigation on the basis of complete clinical records. The first day on which constitutional symptoms were noted was considered the day of onset. Criteria used in making a diagnosis of primary atypical pneumonia were (1) the history and course of the disease, (2) X-ray evidence of pneumonia, (3) a white blood count of less than 12,000 during the first week of illness, and (4) the absence of large numbers of pneumococci in direct sputum examination. Cases in which only small numbers of pneumococci were seen and in which typing was accomplished only after mouse inoculation were not excluded if the course of illness suggested primary atypical pneumonia rather than pneumococcal pneumonia. Five of 14 patients with cases of this type developed significant titers.

Results of Tests. Sera from 156 patients were tested; 73 of 156 convalescent sera were positive, the titers ranging from 10 to 160, with only 11 being 40 or more. Of 41 persons whose convalescent serum was positive, only one showed a significant titer in a specimen taken during the first week of illness. Control tests were done with sera from 32 patients with upper respiratory infections, 54 with pneumococcal or other bacterial pneumonias, and 42 with tuberculosis. None of these had streptococcal agglutinins except two from persons with tuberculosis, in which titers of 10 were found.

Correlation of Results with Severity of the Disease. In an attempt to explain why some patients developed agglutinins while others, whose clinical picture was similar, did not, the results were analyzed with respect to certain clinical features. Two of these, both reflecting the severity of the illness, appeared to show some correlation. These were (1) the duration of fever, and (2) the maximum temperature reading. A similar correlation in the case of cold agglutinins has been suggested by others.[2]

TABLE I.
Correlation of Results of Streptococcus No. 344 Agglutination with Duration of Fever.*

Duration of fever (days)	Positive	Negative	Total	% positive
< 7	7	47	54	13.0
7-12	41	29	70	58.6
>12	25	7	32	78.1
Totals	73	83	156	46.9

* Febrile day defined as one with single oral temperature reading of 99.6°F or higher.

TABLE II.
Analysis of Results of Streptococcus No. 344 Agglutination with Respect to Maximum Temperature.

Max. temp.	Positive	Negative	Total	% positive
<101°F	5	18	23	21.7
101-103°	24	30	54	41.2
>103°	44	34	78	56.4
Totals	73	82	155	47.1

Duration of Fever (Table I). Among patients whose febrile period lasted less than a week, comprising approximately one-third of the total, agglutinins were formed in only 7 of 54 cases. The percentage of positives increased as the length of illness increased. Among those with febrile periods of 12 days or more, 25 of 32 were positive.

Maximum Temperature (Table II). An obvious error enters into this analysis due to the frequent spiking nature of the temperature curve. There appears, nevertheless, to be some correlation. Only 5 of 23 persons whose temperature failed to reach 101°F were positive. On the other hand, of those persons with maximum readings above 103°F, 44 of 78 were positive.

Combined Streptococcus and Cold Agglutination Studies (Table III). Since cold agglutination tests can be done satisfactorily

[2] Finland, M., Unpublished data presented at General Meeting of the Society of American Bacteriologists, New York, N.Y., May 3, 1944.

TABLE III.
Results of Cold Agglutination and Streptococcus No. 344 Agglutination Tests on Sera from 87 Patients with Primary Atypical Pneumonia.

Group	Cold agglutination	Streptococcus No. 344 agglutination	Civilian	Military	Total
I	Positive	Positive	30	6	36
II	"	Negative	14	9	23
III	Negative	Positive	1	2	3
IV	"	Negative	4	21	25
Total			49	38	87

Cold agglutination positive 68%.
Streptococcus No. 344 agglutination positive 45%.

only with fresh serum,[3] the group on which both tests were done was smaller and different in composition. Forty-nine sera were taken from civilian students and 38 from patients in two military establishments. Thirty-six of 39 persons showing streptococcal agglutination also had cold agglutinins. Twenty-three others had cold agglutinins, but no streptococcal agglutinins. Only 3 showed streptococcal agglutinins without cold agglutinins. The proportion negative by both tests was considerably higher in the military group.

Comment. The composition of the group studied differs from that found in most reports on primary atypical pneumonia in that the proportion of moderately severe and severe cases is unusually high. This may account, in part, for the rather high proportion of positive results. In this group of patients the cold agglutination test appeared to be a more sensitive indicator.

The results reported here do not establish the etiological relationship of an indifferent streptococcus to primary atypical pneumonia. This is in accord with the opinion expressed by Thomas and his coworkers.[1] A virus has been isolated from patients with atypical pneumonia,[4] and persons having agglutinins for streptococcus 344 usually have neutralizing antibodies for this virus; but many patients having an increase in neutralizing antibodies associated with atypical pneumonia have no streptococcal agglutinins. The details of these latter observations will be published in a subsequent report.

The authors wish to acknowledge the assistance of the medical and laboratory staffs of Cowell Memorial Hospital, the University of California Hospital, the Santa Ana Army Air Base Station Hospital, and the Camp Roberts Station Hospital.

[3] Meiklejohn, G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 181.

[4] Eaton, M. D., Meiklejohn, G., and van Herick, W., *J. Exp. Med.*, 1944, **79**, 649.

14811 P

Cytoplasmic Particles of Chorio-allantoic Membrane and Their Relations to Purified Preparations of Influenza Virus.

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Ultracentrifugally sedimented preparations of Rous sarcoma virus contain antigenic groupings similar to that of the similarly sedimentable normal tissue components.[1] It would seem desirable that other newly isolated viruses be routinely examined for their content of groupings common to the tissue from

[1] Kabat, E. A., and Furth, J., *J. Exp. Med.*, 1940, **71**, 55.

which they have been derived. The demonstration of apparent physical homogeneity and unique particle size is not sufficient to warrant the assumption of alien chemical uniqueness implicit in the designation of "virus" to the isolated body. This is so because it may be expected that a stage in the synthesis of a virus would require the union of the stimulating virus particle and the protein-synthesizing mechanisms of the host. Thus the liberated particle bearing virus activity may also contain considerable portions of the host, the combination possessing new physical properties. In any case, it may be assumed that the orientation of the chemical work on these virus preparations would be affected if it became clear that a considerable portion of the isolated entity was common to a non-infected host. Virus preparations of influenza* were used as a model for an immunochemical assay, since so much chemical, physical, and biological data have been published on purified preparations.[2,3,4] The virus in the chorioallantoic fluid presumably is liberated into the fluid by the cytolysis of infected cells of the allantoic sac.

The chorioallantoic membranes of normal 11- to 13-day-old chick embryos were rinsed frequently in cold saline containing 0.01% merthiolate and were finely suspended in a final volume of 1 cc per membrane. The suspension was centrifuged at 4000 RPM for 1 hour and the resulting supernate at 30,000 RPM for 20 minutes. The pellets were permitted to soak at 4°C in 0.1 M borate buffer at pH 7.8 for 18 hours and emulsified. The differential centrifugation cycle was repeated and the pellets emulsified in buffer and centrifuged at 4000 RPM to yield an opalescent yellowish solution. A solution at 0.5% concentration was kindly examined in the analytical ultracentrifuge by Dr. M. A. Lauffer of the Rockefeller Institute. The particles, NP, yielded a somewhat diffuse boundary, whose peak had an S_{20} of about 245.† The dialyzed dried particles contained 8.00% nitrogen and 1.76% phosphorus. The average yield per membrane was .05-.06 mg nitrogen in 6 experiments involving 460 membranes.

Rabbit antisera to these materials were found to have fairly high titers of Forssman and particle specific antibodies. The strongest serum was standardized quantitatively by the following method:

The particles, dried over P_2O_5 *in vacuo*, were found to be completely insoluble. Emulsified in saline and placed in normal rabbit sera, these suspensions were quantitatively sedimentable at 2500 RPM for 1 hour. After 2 washings with cold saline, the nitrogen content of the pellet, after a small correction for residual serum nitrogen, was identical with the nitrogen content of NP added. The dried preparations of influenza viruses behaved similarly.

A portion of the antisera to NP was absorbed with sheep red cells. One cc aliquots of saline and of NP suspensions of nitrogen content of .025-.25 mg per cc were added to 1 cc aliquots of normal rabbit sera, the unabsorbed antiserum to NP, and the sheep cell absorbed antiserum. The tubes were frequently mixed during incubation at 37°C for 2 hours and at 4°C overnight. The tubes were centrifuged in the cold at 2500 RPM for 1 hour and the pellets were washed twice with two 1.5 cc aliquots of cold saline. The nitrogen contents were determined and 2 nearly linear curves were obtained when the antibody combined with NP was plotted against antigen nitrogen added. Thus 0.250 mg of NP nitrogen combined with 0.150 mg of antibody nitrogen in the unabsorbed serum and 0.115 mg of antibody nitrogen in the absorbed serum, the difference being Forssman antibody.

The agglutination of suspensions of influenza virus preparations, dialyzed and dried as in the above, was then studied. The preparations were of 3 types: the first was a

* The numerous biological materials were kindly supplied by Dr. W. Henle and Dr. G. Henle of the Children's Hospital of Philadelphia.

2 Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.

3 Stanley, W. M., *J. Exp. Med.*, 1944, **79**, 255.

4 Friedewald, W. F., and Pickels, E. G., *J. Exp. Med.*, 1944, **79**, 301.

† Although this S_{20} is less than one-half that reported for any influenza strain, it is very close to that obtained for equine encephalomyelitis virus from infectious allantoic fluid.

TABLE I.
Estimates of Content of Normal Membrane Particles in Preparations of Influenza Virus.

Virus preparation	Estimations with antisera for normal particles	
	Unabsorbed %	Sheep cell—absorbed %
Multivalent formol vaccine	35	27
PR8	51	57
Chick cell absorbed PR8	29	39

formol-treated preparation of multivalent vaccine, prepared by the ultracentrifugation of the infectious chorioallantoic fluids of the fertile hen's egg, and kindly supplied by Dr. W. M. Stanley of the Rockefeller Institute; the second was a preparation of the PR-8 strain isolated by several differential centrifugation cycles and the third was a preparation of the PR-8 strain concentrated by adsorptions and elutions from chick red blood cells and finally sedimented in the ultracentrifuge.

Saline suspensions were added to the 3 sera employed previously. Absorbed antibody nitrogen was determined, referred to the curves previously estimated, and equivalent NP nitrogen values were obtained. The content of normal allantoic membrane particles was then estimated on the assumption that the normal groupings in the virus preparations were as reactive as if they were present in the normal particles described. These calculations are summarized in Table I.

If these normal particles containing the same antigen distribution were built into the particle bearing virus activity, these estimates are minimal since some normally reactive groups are probably rendered inactive. A number of other possibilities may also limit the validity of these estimates. However, the qualitative conclusion may be drawn that current preparations of the influenza viruses contain antigens typical of the host in amounts sufficiently large to render dubious the significance of chemical data obtained in the process of characterizing this material as an alien and specific viral agent.

14812 P

Motor Analysis of Anti-Peristalsis in the Descending Colon of Rabbit.

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As far as we are aware, antiperistalsis of the descending colon of rabbit has never been studied by direct inspection, yet this can be readily accomplished. The rabbit is narcotized by the subcutaneous injection of 150 mg sodium barbital per kilo; after 45 minutes the descending colon is exposed by a cut through the linea alba from the ensiform cartilage to the pubis; a segment of the descending colon is selected and prepared by making two cuts about 9 cm apart; these cuts sever 60% to 80% of the gut, opposite to the mesenteric border; the segment and adjacent portions of the colon are milked clear of scybala. Now by introducing dry, normal scybala either at the oral or aboral cut, peristaltic and antiperistaltic waves may be produced. Sluggish motor activity is readily improved by subcutaneous injection of 0.1 to 0.3 mg of physostigmine sulfate per kilo.

Results. The introduction of a dry scybalum 1 to 2 cm into the segment at the oral or aboral end generally produces a peristaltic contraction after a short period of time. If the progress of the scybalum is prevented by a gentle digital compression of the segment, the peristaltic wave of contraction sooner or later relaxes and the formerly relaxed, com-

pressed area contracts, the scybalum now moves antiperistaltically and may be expelled at the oral cut or enter the colon oral to the cut.

The antiperistaltic wave shows the following characteristics:

1. A contraction wave of the circularis 0.5 to 1 cm long, produces a grayish-white bloodless cord, while its surface longitudinal layer appears pink. The length of the progressing contraction remains more or less constant. The contraction wave passes without noticeable pause through the cut to the oral colon.

2. A wave of inhibition 1 to 2 cm long precedes the scybalum; this portion of the colon is pink-red, relaxed and shows longitudinal folds; no contraction occurs in this area if it is stroked with a blunt dissecting needle or if digitally compressed for a short time. This inhibitory wave passes the cut and relaxes the colon oral to the cut so that frequently the scybalum enters without difficulty. When alternate peristalsis and antiperistalsis occurs in the segment, then the grayish-white, bloodless, contracted circularis on one side of the scybalum, is seen becoming pink and then relaxing permitting progress of the scybalum, while the circularis on the other side progressively contracts.

Changes seen over the scybalum during its transit: on the face of the scybalum adjacent to the traveling, contracted circularis, a series of 3 to 4 transverse ridges may be seen; they are produced by contraction of the longitudinal fibers and lift the relaxed circularis bands over the scybalum. This is clearly seen when the scybalum enters the cut: in antiperistalsis the aboral lip of the oral cut is curled back 3 mm plus by the contraction of the longitudinal fibers and the relaxed, inhibited, circularis is lifted over the scybalum, and then the circularis contracts and the scybalum enters the inhibited colon oral to the cut. After its entry the circularis of this section contracts powerfully; the oral lip of the cut, however, shows but little curling, usually 1 plus mm. Inspection of the lips of a cut shows thus whether a peristaltic or antiperistaltic wave has recently passed.

The speed of the antiperistaltic wave is much slower than that of a peristaltic wave.

No clear-cut antiperistalsis was ever seen after pithing the spinal cord, combined with subdiaphragmatic section of both splanchnics and vagi.

Antiperistalsis in our experiments, therefore, obeys the same basic conditions developed by Bayliss and Starling[1] for peristalsis, if allowance is made for the change in direction.

1 Bayliss, W. W., and Starling, E. H., *J. Physiol.*, 1899, **24**, 99; *ibid.*, 1900, **26**, 107 and 125.

14813 P

Inhibition of Choline Acetylase by α-Keto Acids.*

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Evidence has been provided for the assumption that the energy released by the breakdown of phosphocreatine is adequate to account for the electric energy released by the nerve action potential.[1] Hence if the primary event responsible for the alterations of the nerve membrane during the passage of the impulse is the release of acetylcholine, as recently suggested,[2-4] energy rich phosphate

* This work was made possible by grants of the Josiah Macy, Jr., and Dazian Foundations.

1 Nachmansohn, D., Cox, R. T., Coates, C. W., and Machado, A. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 97; *J. Neurophysiol.*, 1943, **6**, 383.

2 Nachmansohn, D., Cox, R. T., Coates, C. W., and Machado, A. L., *J. Neurophysiol.*, 1942, **5**, 499.

3 Nachmansohn, D., *Collecting Net*, 1942, **17**, 61; *Biol. Bull.*, 1944, **87**, 158.

TABLE I.
Inhibitory Effect of α-ketoacids on Choline Acetylase Prepared from Fresh Brain or from Acetone Dried Powder (1 g of powder = 5-6 g of fresh brain).

Compound	Extracts from fresh brain		Extracts from dried brain	
	Conc., M × 10⁻⁴	ACh formed μg/g/hr	Conc., M	ACh formed μg/g/hr
Pyruvic acid	0.0	108	0.0	600
	3.15	63	2×10^{-3}	440
	6.3	49		
α-Keto glutaric acid	0.0	128	0.0	390
	3.15	101	1.25×10^{-4}	200
	6.3	85	2.5×10^{-4}	160
Oxyphenyl pyruvic acid	0.0	155	control	820
	2.5	102	no eserine	780
	5.0	93	no fluoride	810

bonds should be used for reversing the process and rebuilding the ester. In accordance with this hypothesis an enzyme, choline acetylase, has been extracted from brain which in presence of adenosine-triphosphate under strictly anaerobic conditions synthesizes acetylcholine in cell free solution. From 1 g fresh rat brain an enzyme solution may be prepared which forms 100-150 μg of acetylcholine (ACh) per hour. Presence of eserine and fluoride is necessary to inhibit the action of choline esterase and adenosinetriphosphatase. The enzyme contains active sulfhydryl groups which may be easily oxidized and are readily inactivated by monoiodoacetic acid or Cu in low concentrations.[5] On dialysis choline acetylase rapidly loses its activity. Addition of the natural *l* (+) glutamic acid reactivates it partly.[6] With potassium + glutamic acid 50-80% of the original activity may be restored; further addition of cyanide or replacement of glutamic acid by cysteine may reactivate the enzyme nearly completely.[7,8] The experiments suggest that the enzyme may require besides active –SH groups and potassium the presence of an amino acid group.

It has now been found that oxidized amino acids, *i.e.*, α-keto acids, are strong inhibitors of choline acetylase. Pyruvic acid, α-ketoglutaric acid, phenyl- and oxyphenyl pyruvic acid have been tested so far. They inhibit choline acetylase in concentrations of 10^{-3} to 10^{-4} M. Table I gives a few significant data. The effect has also been tested on extracts prepared from powder of acetone dried brain. In these extracts the enzyme is about twice as pure as in those obtained from fresh tissue, 1 g of protein forming 3-4 mg of ACh in 60 min; the choline acetylase is separated from adenosinetriphosphatase so that no addition of fluoride is required;[7] choline esterase is largely or sometimes almost completely removed so that addition of eserine has either a small effect or practically no effect. Two observations are reproduced in the table; the last experiment recorded shows the small effect of eserine and fluoride.

α-keto acids occur in the living cell in concentrations close to those found to have an inhibitory effect. Moreover, pyruvic acid is known to be a "physiological anticonvulsant."[9] The strong inhibitory effect of these compounds on choline acetylase appears therefore to be of general physiological as well as of clinical interest.

[4] Fulton, J. F., and Nachmansohn, D., *Science*, 1943, **97**, 569.

[5] Nachmansohn, D., and Machado, A. L., *J. Neurophysiol.*, 1943, **6**, 397.

[6] Nachmansohn, D., John, H. M., and Waelsch, H., *J. Biol. Chem.*, 1943, **150**, 485.

[7] Nachmansohn, D., and John, H. M., *J. Biol. Chem.*, in press.

[8] Nachmansohn, D., *Vitamins and Hormones*, in press.

[9] Putnam, T. J., and Merritt, H. H., *Arch. Neurol. and Psych.*, 1941, **45**, 505.

14814

On the Growth Requirements of Pneumococci.

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A semi-synthetic medium developed for the CHA strain of type III pneumococcus[1] failed to support optimal growth of other types and strains. This led to a study of the nutritional requirements of different strains and resulted in certain modifications of the semi-synthetic medium. This modified medium, described below, supported the growth of some 25 strains other than the original CHA pneumococcus.

Preparation of the Medium. The constituents of the improved medium, listed in Table I, are combined as follows. The cystine, adenine, guanine, and uracil are dissolved in the acid casein hydrolysate by heating slightly. This solution is diluted with 500 ml distilled water after which the acetate, $MgSO_4$, and KH_2PO_4 are added. The pH is adjusted to 7.6 to 7.8 with NaOH and the volume made up to 800 ml. This basal medium is then heated to boiling, filtered while hot through filter paper, and aged in the refrigerator at least 3 days before the growth factors are added.

TABLE I.
Constituents of the CHA and the Improved Media for Pneumococci.

	CHA medium	Improved medium
Casein hydrolysate*	0.5 g N	1.25 g N
l-Cystine	25.0 mg	20.0 mg
Adenine sulfate		10.0 "
Guanine hydrochloride		10.0 "
Uracil		10.0 "
$NaC_2H_3O_2 \cdot 3H_2O$		200.0 "
$MgSO_4 \cdot 7H_2O$	400.0 "	400.0 "
KH_2PO_4	5.0 g	6.0 g
Biotin	2.0 μg	2.0 μg
Choline chloride	5.0 mg	6.0 mg
Calcium pantothenate	1.0 "	2.5 "
Nicotinic acid	1.0 "	5.0 "
Thiamin hydrochloride	2.0 "	2.0 "
Asparagine	1.0 "	1.0 "
Ascorbic acid	300.0 "	350.0 "
Glucose†	5.0 g	5.0 g
Distilled water to make 1 liter		

* 500 g SMACO Vitamin-Free Casein was hydrolyzed for 18 hours in 3 liters of 1:1 HCl. The hydrolysate was concentrated twice *in vacuo*, diluted to 2.5 liters, and treated with Darco charcoal until water clear (about 5 times with 50 g charcoal).

† Autoclaved separately and added aseptically after medium is autoclaved.

To the aged basal medium are added the choline, nicotonic acid, pantothenate, biotin, thiamin, asparagine, and ascorbic acid. Except for the ascorbic acid, these growth factors are added from stock solutions kept in the refrigerator. The volume is made up to 1000 ml with distilled water, and the resulting medium is tubed in 5 ml portions and autoclaved at 15 lb for 10 minutes. 0.1 ml of a 25% solution of sterile glucose is added to each tube which is then carefully shaken to insure homogeneity. The complete medium is kept in the dark at 5 to 9°C until it is used.

Organisms and Inoculum. Throughout the major part of this investigation 5 strains of pneumococci were employed, including SV-1 and McGovern strains of type I, the CH strain of type II and Wistuba and CHA strains of type III. For a period of 7 years these strains had been passed through mice at least every other day and cultured in beef-infusion broth enriched with 2% defibrinated rabbit blood. The other strains tested had been stored 2 to 5 years in a dehydrated state. Prior to testing for growth in the improved medium, these latter strains were cultured in beef-infusion broth enriched with blood.

The inoculum was prepared by subculturing one drop of the stock culture in 5 ml of the semi-synthetic medium and incubating 5 hours at 37°C. This young culture was centrifuged and the cells washed once and diluted 1000 times in sterile basal medium. 0.1 ml of this dilution added to each tube gave an inoculum of 1000 to 10,000 organisms per ml. The inoculated tubes were incubated at 37°C and turbidity resulting from growth was measured

* Christ Hospital Fellow in Biological Chemistry, University of Cincinnati.

1 Badger, E., *J. Bact.*, 1944, **47**, 509.

TABLE II.
Effect of Adenine, Guanine, and Uracil on the Growth of Pneumococci.

Purine or pyrimidine*	Turbidity readings after 12 and 14 hrs incubation									
	I SV		I McGovern		II CH		III Wistuba		III CHA	
	12	14	12	14	12	14	12	14	12	14
None	6†	79	12	22	0‡	0	8	64	51	135
Adenine	45	170	30	125	0	0	27	168	106	179
Guanine	98	170	30	104	0	0	152	172	81	150
Uracil	8	81	12	24	0	40	99	183	65	153
Adenine + guanine	103	182	25	113	0	11	164	176	147	170
" + uracil	70	174	37	160	143	159	164	185	74	155
Uracil + guanine	126	186	122	151	67	143	178	188	95	157
Adenine + " + uracil	133	192	115	158	115	145	177	190	159	175

* Added to basal medium as adenine sulfate, guanine hydrochloride and uracil; 10 μg per ml.
† Uninoculated medium reads zero.
‡ Grew out after 24 hours' incubation.

in a Klett-Summerson photoelectric colorimeter equipped with a 540 mμ filter.

Response of the 5 Strains to Modifications of the CHA Medium. In Table I are listed the constituents of the CHA medium and the improved medium. A comparison of the 2 media shows, first of all, certain changes in concentrations of the original constituents. These changes were instituted after the following observations were made. Better growth of all strains was obtained by increasing the casein hydrolysate from 0.5 to 1.25 mg N per ml. Lowering the concentration of cystine from 25 to 20 μg and raising the concentration of ascorbic acid from 300 to 350 μg per ml provided better growth of II CH but had no effect on the other strains. Raising the concentration of $KH_2PO_4^-$ from 5 to 6 mg per ml increased the rate of growth of Wistuba, II CH, and McGovern. It was not possible to replace the KH_2PO_4 with sodium glycerophosphate.

The minimum effective concentration of each of the 4 essential growth factors, choline, nicotinic acid, pantothenic acid and biotin agreed with those determined for CHA.[1] However, to insure the presence of an excess but still optimal concentration of the growth factors, choline chloride was raised from 5 to 6 μg per ml, calcium pantothenate from 1 to 2.5 μg per ml, and nicotinic acid from 1 to 5 μg per ml.

The second difference in the 2 media is the addition of adenine, guanine, uracil, and acetate. It was found that adenine, guanine, and uracil are not essential for growth, but they do have an accelerating effect, which is most apparent in the early hours of incubation and with small inocula. They also appear to be more effective when added to the basal medium than when added later with the growth factors, suggesting an interaction with the basal medium constituents. These factors probably explain why the accelerating effect of adenine, guanine, and uracil was not observed in the original studies of CHA. From the data in Table II it is seen that the response to the individual purines and the pyrimidine and their various combinations differs with each strain. Type III CHA seemed least affected by any combination, while the growth of II CH was markedly accelerated by the addition of adenine and uracil but was inhibited by guanine.

The addition of synthetic thymine† alone or in combination with adenine, guanine, and/or uracil produced no measurable effect. Xanthine gave no further acceleration of growth in the presence of all 3 but could partially replace guanine. Bohonos and Subbarow[2] have also included adenine, guanilic acid, and uracil in their medium for pneumococcus.

The accelerating action of sodium acetate was observed during preliminary studies of the effect of alkaline-treated peptone on the growth of pneumococcus. The addition of

† We are indebted to Dr. J. L. Stokes of Merck and Co., Inc., for the thymine.

2 Bohonos, N., and Subbarow, Y., *Arch. Biochem.*, 1943, **3**, 257.

sodium acetate in a concentration of 100 μg per ml of medium accelerated the growth of CHA and Wistuba. The response of strain Wistuba to sodium acetate is shown in Table III. That this accelerating effect is not due to a buffering action is apparent from the relatively small concentration required as compared to the phosphate present. Neutralized glacial acetic acid is as effective as the sodium acetate, suggesting that the acetate radical is the active factor rather than some contaminating ion. The acetate could not be replaced by succinate, lactate, citrate, pyruvate, or glycine. Further study is indicated on the role of acetate in pneumococcal metabolism.

TABLE III.
The Response of Type III Strain Wistuba to Sodium Acetate.

Sodium acetate μg per ml	Turbidity readings*
0	77
10	103
50	128
100	175
200	175
500	133

* Readings made after 12 hours' incubation.

The response of the pneumococci to thiamin and asparagine varied. Thiamin accelerated growth of all 5 strains when present in a concentration of 1 to 3 μg per ml. The effect on the growth of strains II CH, CHA, and Wistuba was much more pronounced than upon the two type I strains. These type I strains were also least affected by asparagine as is seen in Table IV. From the data presented here and similar experiments the optimum concentration of asparagine is seen to be 1 μg per ml. This optimum concentration is quite sharp, since 0.5 or 1.5 μg per ml provides slower growth. Concentrations greater than 2 μg are definitely inhibitory for all 5 strains. This concentration of asparagine is exceedingly small compared with that used by Bohonos and Subbarow[2] or Roe and Adams,[3] who included asparagine in their media for pneumococcus in a concentration of 250 and 100 μg per ml respectively.

That asparagine cannot be replaced by glutamine is also shown in Table IV. In high concentrations glutamine stimulated the growth of II CH, Wistuba, and CHA, but inhibited the growth of the two type I strains. When the asparagine and glutamine were sterilized by filtration the results were the same as given in Table IV. With *Streptococcus lactis*, Niven[4] found that asparagine was more satisfactory in casein hydrolysate media, while glutamine was more effective in media containing pure amino acids.

Growth of Other Strains in the Improved Medium. Having developed a simplified medium for the 5 strains of pneumococci mentioned above, it was interesting to test its ability to support the growth of other strains. Twenty-four additional strains, not recently passed through mice, were tested in the improved medium. Growth after 5 successive subcultures from an initial small washed inoculum was obtained with 21 out of 24 strains. Those that gave an early rapid growth included types I Lederle, I 1811, IV NYS, V NYC, VI NYC, IX Ramey, XI NYC, XIX NYS, XXIII NYS, XXIII Marvin, XXVII NYC, XXIX NYS, and XXXII NYC; while those which grew out more slowly included types II 2118, III H, VII CHA, XIV Halse, XV Leichliter, XXVII Glacken, XXXI NYS, and XXXIII League. Strains XXXI NYC, X NYC, and X NYS failed to grow out in 48 hours. However, slow growth of the two type X strains was obtained in the medium without guanine.

Variation in the growth requirements of the strains of pneumococci was indicated by the different rates of growth in the simplified medium. Some of the factors affecting this variation were demonstrated in an experiment in which growth in the complete medium was compared with that in the medium from which one of the growth factors had beeen omitted. The results of this study are summarized in Table V. The omission of a factor resulted in either (1) no growth in 48 hours, (2) delayed growth, (3) growth the same as the control, or (4) improved accelerated growth. All strains tested required biotin, choline, nicotinic acid, and pantothenic acid for growth,

[3] Roe, A. S., and Adams, M. H., *J. Bact.*, 1944, **47**, 432.

[4] Niven, C. F., *J. Bact.*, 1944, **47**, 343.

TABLE IV.
Response of Pneumococci to Glutamine and Asparagine.

Glutamine or asparagine	μg per ml	Turbidity readings*				
		I SV	I McGovern	II CH	III Wistuba	III CHA
None		85	135	0	11	71
Asparagine	0.1	69	125	0	60	90
	1.0	80	125	85	185	102
	2.0	60	100	25	170	85
	10.0	0	20	0	36	0
	100.0	0	14	0	0	0
Glutamine	0.1	85	90	0	0	85
	1.0	80	93	0	0	87
	10.0	7	45	0	16	85
	100.0	0	27	65	41	107

* Readings made after 12 hours' incubation; uninoculated medium reads zero.

TABLE V.
Effect of Omitting Certain Factors from the Complete Medium on Growth of 26 Strains of Pneumococci.

Factor omitted	No growth*	Delayed growth	Growth not affected	Accelerated growth
Biotin	26			
Choline	26			
Nicotinic acid	26			
Pantothenic acid	26			
Asparagine	6	12	3	5
Thiamin		24		2
Adenine		21	1	4
Guanine		20	4	2
Uracil	3	14	3	6

* No growth after 48 hours' incubation.

confirming the observations of Rane and Subbarow[5] and Bohonos and Subbarow[2] with other strains. The effect of asparagine, thiamin, adenine, guanine, and uracil on the growth of these pneumococci varied from being essential for growth of some strains to actually inhibiting the growth of others.

That this improved medium is still incomplete is indicated by the accelerating action of small amounts of peptone. Of the growth-stimulating substances tested, none was able to replace this peptone action.

[5] Rane, L., and Subbarow, Y., *J. Bact.*, 1940, 40, 695.

Conclusions. A simplified medium has been described which supported the growth of 26 out of 29 strains of pneumococci. All strains required biotin, choline, nicotinic acid, and pantothenic acid for growth, but varied in their response to thiamin, asparagine, adenine, guanine, and uracil. The extent of this variation makes apparent the necessity of considering the growth requirements of each strain in the study of pneumococcal nutrition. In such a study the importance of concentration and interaction of medium constituents, size of inoculum, and the time of measurement of growth cannot be overemphasized.

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Modifications in Feather Pattern and Growth Rate Following Administration of Thiouracil in Brown Leghorn Fowl.*

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The administration of adequate doses of thiourea, thiouracil, and related compounds in various mammals, particularly the rat, is followed by marked hyperplasia and hypertrophy of the thyroid gland accompanied by signs and symptoms of hypothyroidism.[1,2] In chicks fed sulfaguanidine[2] there was neither hypertrophy nor inhibition of the thyroid, but feeding of thiourea and thiouracil resulted in its hypertrophy.[3]

In the Brown Leghorn fowl hypothyroidism is manifested in the plumage by a decrease in barbulation and by the replacement of melanin with a red pigment resulting in a reddish, narrow, lacy feather.[4,5] These changes are most evident on the breast, lateral and ventral areas where normally occurs a black, broad, webbed feather. The characteristic growth rates of the various feather areas are noticeably reduced.[6] Since some of the manifestations of hypothyroidism in plumage are now well established, experiments were devised in an effort to determine whether this condition could be produced in the fowl by the administration of certain of these goitrogenic compounds. In the following report the reaction to thiouracil in Brown Leghorn capons and cockerels is described, employing the plumage as an indicator.

Methods. Measured amounts of thiouracil were administered daily as a dry powder contained in a gelatin capsule which was placed on the back of the tongue and palpated down the esophagus. In most previous experiments employing these drugs, and in all using the chicken as a test animal, dosage was approximated since the drug was administered *ad libitum* by mixing it with food. In order to determine the period when effects on plumage first become evident, semi-weekly microscopic examinations of split, regenerating feather germ preparations were made. Feather growth rates were studied by measurements made on alternate days in capons and every sixth day in cockerels. Comparable areas were selected on back, saddle, and anterior and posterior breast feather tracts and 25 regenerating feathers per tract were measured in each bird.

Results. Daily administration of 0.4 g of thiouracil was begun in an adult capon 6 days after plucking. The first definite indication of an effect was noted after 26 days of medication when regenerating breast feather germs revealed a basal deposition of red pigment. Red pigment deposition continued and 48 days later when medication was discontinued (total 74 days) emerging breast feathers were red and showed a reduction in barbulation. Feather growth rates at this time were noticeably reduced in all areas. After emergence, breast feathers were red throughout and exhibited a fluffy or lacy condition instead of the characteristic black color and broad webbing (Fig. 1). Back and saddle feathers showed an increase in red pigment and some reduction in barbulation producing a feather which was narrower and somewhat more lacy than normal. By 18 to 19 days following cessa-

* This investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago. Grateful acknowledgment is made to Dr. George F. Cartland, The Upjohn Company, for the thiouracil used in these experiments.

1 Astwood, E. B., Sullivan, J., Bissell, A., and Tyslowitz, R., *Endocrinol.*, 1943, **32**, 210.

2 MacKenzie, C. G., and MacKenzie, J. B., *Endocrinol.*, 1943, **32**, 185.

3 Mixner, J. P., Reineke, E. P., and Turner, C. W., *Endocrinol.*, 1944, **34**, 168.

4 Greenwood, A. W., and Blyth, J. S. S., *Proc. Roy. Soc. Edinburgh*, 1929, **49**, 313.

5 Blivaiss, B. B., and Domm, L. V., *Anat. Rec.*, 1942, **84** (suppl.), 79.

6 Blivaiss, B. B., and Domm, L. V., *Anat. Rec.*, 1944, **89** (suppl.), 37.

FIG. 1.
Capon breast feathers. Right normal. It is black except for basal fluff and shows a solid web-like vane. Left from thiouracil-treated capon. The feather is red anteriorly and lacy in the normally webbed area due to reduction in barbulation. The basal fluff developed after medication was discontinued is similar in character to the normal.

tion of medication developing breast feather germs again showed deposition of black pigment and posterior breast feather measurements revealed accelerated growth.

In a second adult capon receiving 0.5 g of thiouracil daily, results were essentially similar to those described above.

The first indication of an effect was noticed on regenerating breast feather germs 24 days after medication began, at which time they showed a narrow, irregular band of red at the base. Medication was continued for 75 days, when emerging breast feathers were red and lacy and growth rates were below controls in all areas. Twenty days after medication ceased developing breast feathers again showed black pigment and increased barbulation.

In a third experiment, four 5-months-old cockerels received daily medication of 0.3 g of thiouracil. Feather areas on the right side were deplumed on the day treatments began. Beginning on the 25th day of treatment dosage was increased to 0.4 g and feather areas on left side were plucked. After 30 days total medication ingrowing feathers on right side, now several centimeters long in all deplumed areas, showed no significant modification in color or form but did show a decline in growth rate as compared with controls. However, microscopic examination of split breast feather germs at this time revealed a basal deposition of red pigment. This process has continued producing red, lacy feathers in plucked areas similar to those developed in the treated capon. A period of 28 to 30 days medication was thus found necessary to produce these changes in the plumage of the cockerel. A single injection of 1.0 mg thyroxin brought about a deposition of melinin and increased barbulation in growing breast feathers.

Summary. Daily administration of 0.3 to 0.5 g of thiouracil in Brown Leghorn capons and cockerels brought about changes in plumage similar to those observed following thyroidectomy in this breed. Black pigments were replaced by red, barbulation was reduced, and growth rates retarded. Changes in feather color were first apparent 24 to 28 days following initiation of medication and in capons continued for approximately 18 to 20 days after medication ceased.

In a recent note[7] Juhn reported similar modifications of breast feather pattern in the Brown Leghorn capon following thiouracil administration *ad libitum* by mixing with food. No record was made of observations on growth rate.

[7] *Endocrinol.*, **35**, 278.

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